

REMARKS

Amendments

Claims 1-27, 32 and 34 have been canceled; claims 28-31, 33, 35, 38 and 40 have been amended, and claims 28-31, 33 and 35-40 are pending in the application. No new matter is added by the amendments.

The foregoing amendments are made solely to expedite prosecution of the application and are not intended to limit the scope of the invention. Further, the amendments to the claims are made without prejudice to the pending or now canceled claims or to any subject matter pursued in a related application. The Applicant reserves the right to prosecute any canceled subject matter at a later time or in a later filed divisional, continuation, or continuation-in-part application. Entry of the Amendment and reconsideration of the claims in view of the following Remarks is respectfully requested.

Rejections

Rejections under 35 U.S.C. § 101

Claims 28-34 were rejected under 35 U.S.C. 101 for alleged lack of patentable utility. The Applicant respectfully traverses this rejection.

Applicant respectfully traverses the rejection. Amended claim 35 is drawn to a transgenic mouse whose genome comprises a null endogenous CASH allele. According to 35 U.S.C. § 101, “[w]hoever invents . . . any new and useful . . . composition of matter may obtain a patent therefore. . . . “

Under the Patent Office’s Utility Requirement Guidelines:

If at any time during the examination, it becomes readily apparent that the claimed invention has a well-established utility, do not impose a rejection based on lack of utility. An invention has a well-established utility if (i) a person of ordinary skill in the art would immediately appreciate why the invention is useful based on the characteristics of the invention (e.g., properties or applications of a product or process), and (ii) the utility is specific, substantial, and credible.

. . .

If the applicant has asserted that the claimed invention is useful for any particular practical purpose (i.e., it has a “specific and substantial utility”) and the assertion would be considered credible by a person of ordinary skill in the art, do not impose a rejection based on lack of utility.

(emphasis added)(MPEP § 2107, II (A)(3); II (B)(1)).

The standard for “credible” is defined as:

... whether the assertion of utility is believable to a person of ordinary skill in the art based on the totality of evidence and reasoning provided. An assertion is credible unless (A) the logic underlying the assertion is seriously flawed, or (B) the facts upon which the assertion is based are inconsistent with the logic underlying the assertion.

(MPEP 2107.02, III(B)(emphasis added).

According to the Patent Office’s own guidance to Examiners:

Langer and subsequent cases direct the Office to presume that a statement of utility made by an applicant is true. [citations omitted] ... Clearly, Office personnel should not begin an evaluation of utility by assuming that an asserted utility is likely to be false.

Compliance with 35 U.S.C. 101 is a question of fact [citations omitted]. Thus, to overcome the presumption of truth that an assertion of utility by the applicant enjoys, Office personnel must establish that it is more likely than not that one of ordinary skill in the art would doubt (i.e., “question”) the truth of the statement of utility. ... To do this, Office personnel must provide evidence sufficient to show that the statement of asserted utility would be considered “false” by a person of ordinary skill in the art.

(MPEP 2107.02, III(A)(emphasis added).

Rejections under 35 U.S.C. 101 have been rarely sustained by federal courts.

Generally speaking, in these rare cases, the 35 U.S.C. 101 rejection was sustained either because the applicant failed to disclose any utility for the invention or asserted a utility that could only be true if it violated a scientific principle, such as the second law of thermodynamics, or a law of nature, or was wholly inconsistent with contemporary knowledge in the art. *In re Gazave*, 379 F.2d 973, 978, 154 USPQ 92, 96 (CCPA 1967). Special care therefore should be taken when assessing the credibility of an asserted therapeutic utility for a claimed invention. In such cases, a previous lack of success in treating a disease or condition, of the absence of a proven animal model for testing the effectiveness of drugs for treating a disorder in humans, should not, standing alone, serve as a basis for challenging the asserted utility under 35 U.S.C. 101.

(MPEP 2107.02, III(B)(emphasis in original and added). The Guidelines additionally provide that:

There is no predetermined amount or character of evidence that must be provided by an applicant to support an asserted utility, therapeutic or otherwise. Rather, the character and amount of evidence needed to support an asserted utility will vary depending on what is claimed (citations omitted), and whether the asserted utility appears to contravene established scientific principles and beliefs. (citations omitted). Furthermore, the

applicant does not have to provide evidence sufficient to establish that an asserted utility is true “beyond a reasonable doubt.” (citations omitted). Nor must an applicant provide evidence such that it establishes an asserted utility as a matter of statistical certainty. Nelson v. Bowler, 626 F.2d 853, 856-57, 206 USPQ 881, 883-84 (CCPA 1980)(reversing the Board and rejecting Bowler’s arguments that the evidence of utility was statistically insignificant. The court pointed out that a rigorous correlation is not necessary when the test is reasonably predictive of the response).

(MPEP 2107.02, VII)(emphasis added).

Thus, according to Patent Office guidelines, a rejection for lack of utility may not be imposed where an invention has a well-established utility or is useful for any particular practical purpose. An assertion of utility is presumed to be true. The burden is on the Examiner to show that one of ordinary skill would find the asserted utility to be false. The present invention satisfies either standard.

The present invention has a well-established utility since a person of ordinary skill in the art “would immediately appreciate why” knockout mice are useful. As a general principle, knockout mice have the inherent and well-established utility of defining the function and role of the disrupted target gene, regardless of whether the inventor has described any specific phenotypes, characterizations or properties of the knockout mouse. The sequencing of the human genome has produced countless genes whose function has yet to be determined.

According to the National Institute of Health, knockout mice represent a critical tool in studying gene function:

Over the past century, the mouse has developed into the premier mammalian model system for genetic research. Scientists from a wide range of biomedical fields have gravitated to the mouse because of its close genetic and physiological similarities to humans, as well as the ease with which its genome can be manipulated and analyzed.

...

In recent decades, researchers have utilized an array of innovative genetic technologies to produce custom-made mouse models for a wide array of specific diseases, as well as to study the function of targeted genes. One of the most important advances has been the ability to create transgenic mice, in which a new gene is inserted into the animal's germline. Even more powerful approaches, dependent on homologous recombination, have permitted the development of tools to “knock out” genes, which involves replacing existing genes with altered versions; or to “knock in” genes, which involves altering a mouse gene in its natural location. To preserve these extremely valuable strains of mice and to assist in the propagation of strains with poor reproduction, researchers have taken

advantage of state-of-the-art reproductive technologies, including cryopreservation of embryos, in vitro fertilization and ovary transplantation.

(<http://www.genome.gov/pfv.cfm?pageid=10005834>)(emphasis added)(copy attached).

Thus, the knockout mouse has been accepted by the NIH as the premier model for determining gene function, a utility that is specific, substantial and credible.

Knockout mice are so well accepted as tools for determining gene function that the director of the NIH Chemical Genomics Center of the National Human Genome Research Institute (among others, including Capecchi, Bradley, Joyner, Nagy and Skarnes) has proposed creating knockout mice for all mouse genes:

Now that the human and mouse genome sequences are known, attention has turned to elucidating gene function and identifying gene products that might have therapeutic value. The laboratory mouse (*Mus musculus*) has had a prominent role in the study of human disease mechanisms throughout the rich, 100-year history of classical mouse genetics, exemplified by the lessons learned from naturally occurring mutants such as agouti, reeler and obese. The large-scale production and analysis of induced genetic mutations in worms, flies, zebrafish and mice have greatly accelerated the understanding of gene function in these organisms. Among the model organisms, the mouse offers particular advantages for the study of human biology and disease: (i) the mouse is a mammal, and its development, body plan, physiology, behavior and diseases have much in common with those of humans; (ii) almost all (99%) mouse genes have homologs in humans; and (iii) the mouse genome supports targeted mutagenesis in specific genes by homologous recombination in embryonic stem (ES) cells, allowing genes to be altered efficiently and precisely.

...

A coordinated project to systematically knock out all mouse genes is likely to be of enormous benefit to the research community, given the demonstrated power of knockout mice to elucidate gene function, the frequency of unpredicted phenotypes in knockout mice, the potential economies of scale in an organized and carefully planned project, and the high cost and lack of availability of knockout mice being made in current efforts.

(Austin et al., Nature Genetics (2004) 36(9):921-24, 921)(emphasis added)(copy attached).

With respect to claims drawn to transgenic mice having a null allele, the following comments from Austin are relevant:

Null-reporter alleles should be created

The project should generate alleles that are as uniform as possible, to allow efficient production and comparison of mouse phenotypes. The alleles should achieve a balance of utility, flexibility, throughput and cost. A null allele is an indispensable starting point for studying the function of every gene. Inserting a reporter gene (e.g., P-galactosidase

or green fluorescent protein) allows a rapid assessment of which cell types normally support the expression of that gene.

(p. 922)(emphasis in original, emphasis added).

Research tools such as knockout mice are clearly patentable, as noted by the Patent

Office:

Some confusion can result when one attempts to label certain types of inventions as not being capable of having a specific and substantial utility based on the setting in which the invention is to be used. One example is inventions to be used in a research or laboratory setting. Many research tools such as gas chromatographs, screening assays, and nucleotide sequencing techniques have a clear, specific and unquestionable utility (e.g., they are useful in analyzing compounds). An assessment that focuses on whether an invention is useful only in a research setting thus does not address whether the invention is in fact “useful” in a patent sense. Instead, Office personnel must distinguish between inventions that have a specifically identified substantial utility and inventions whose asserted utility requires further research to identify or reasonably confirm. Labels such as “research tool,” “intermediate” or “for research purposes” are not helpful in determining if an applicant has identified a specific and substantial utility for the invention.

(MPEP § 2107.01, I). As with gas chromatographs, screening assays and nucleotide sequencing techniques, knockout mice have a clear, specific and unquestionable utility (e.g., they are useful in analyzing gene function), one that is clearly recognized by those skilled in the art.

For example, according to the Molecular Biology of the Cell (Albert, 4th ed., Garland Science (2002)) (copy of relevant pages attached), one of the leading textbooks in the field of molecular biology:

Extensive collaborative efforts are underway to generate comprehensive libraries of mutation in several model organisms including . . . the mouse. The ultimate goal in each case is to produce a collection of mutant strains in which every gene in the organism has either been systematically deleted, or altered such that it can be conditionally disrupted. Collections of this type will provide an invaluable tool for investigating gene function on a genomic scale.

(p. 543)(emphasis added).

According to Genes VII (Lewin, Oxford University Press (2000)) (copy of relevant pages attached), another well respected textbook in the field of genetics:

The converse of the introduction of new genes is the ability to disrupt specific endogenous genes. Additional DNA can be introduced within a gene to prevent its

expression and to generate a null allele. Breeding from an animal with a null allele can generate a homozygous “knockout”, which has no active copy of the gene. This is a powerful method to investigate directly the importance and function of the gene.

(p. 508)(emphasis added).

According to Joyner (Gene Targeting: *A Practical Approach*, Oxford University Press 2000) (copy of relevant pages attached),:

Gene targeting in ES cells offers a powerful approach to study gene function in a mammalian organism.

(preface)(emphasis added).

According to Matisse et al. (*Production of Targeted Embryonic Stem Cell Clones* in Joyner, Gene Targeting: *A Practical Approach*, Oxford University Press 2000)(copy of relevant pages attached):

The discovery that cloned DNA introduced into tissue culture cells can undergo homologous recombination at specific chromosomal loci has revolutionized our ability to study gene function in cell culture and *in vivo*. . . . Thus, applying gene targeting technology to ES cells in culture affords researchers the opportunity to modify endogenous genes and study their function *in vivo*.

(p. 101)(emphasis added).

According to Crawley (What’s Wrong With My Mouse *Behavioral Phenotyping of Transgenic and Knockout Mice*, Wiley-Liss 2000) (copy of relevant pages attached):

Targeted gene mutation in mice represents a new technology that is revolutionizing biomedical research.

Transgenic and knockout mutations provide an important means for understanding gene function, as well as for developing therapies for genetic diseases.

(p. 1, rear cover)(emphasis added).

In addition, commercial use and acceptance is an important indication that the utility of an invention has been recognized by one of skill in the art (“A patent system must be related to the world of commerce rather than to the realm of philosophy.” *Brenner v Manson*, 383 U.S. 519, 148 U.S.P.Q. 689, 696 (1966)). Commercial use of the knockout mice produced by Assignee Deltagen has been clearly established. The claimed mouse has been extensively analyzed using the tests set forth in the Examples. This data has been incorporated into

Deltagen's commercial database product, DeltaBase. This database has been subscribed to by at least three of the world's largest pharmaceutical companies, Merck, Pfizer and GSK. In addition, the present invention has been delivered to at least one (1) large pharmaceutical company. This acceptance more than satisfies the practical utility requirement of section 101 as it cannot be reasonably argued that a claimed invention which is actually being used by those skilled in the art has no "real world" use. (see, for example, Phillips Petroleum Co. v. U.S. Steel Corp., 673 F. Supp. 1278, 6 U.S.P.Q.2d 1065, 1104 (D. Del. 1987), *aff'd*, 865 F.2d 1247, 9 U.S.P.Q.2d 1461 (Fed. Cir. 1980)("lack of practical utility cannot co-exist with infringement and commercial success"); (Lipscomb's Walker on Patents, §5:17, p. 562 (1984)("Utility may be evidenced by sales and commercial demand."))

As evidence of such sales and purpose of such use, attached hereto is a Rule 132 Declaration from Robert Driscoll, Vice President of Intellectual Property & Legal Affairs of Assignee, Deltagen.

Applicant submits that since one of ordinary skill in the art would immediately recognize the utility of a knockout mouse in studying gene function, a utility that is specific, substantial and credible, the invention has a well-established utility, thus satisfying the utility requirement of section 101. On this basis alone, withdrawal of the rejection with respect to the present invention is warranted, and respectfully requested.

In addition, the claimed invention is useful for a particular purpose. The Applicant has demonstrated and disclosed specific phenotypes of the presently claimed mice. Utility of the claimed knockout mouse would be apparent to, and considered credible by, one of skill in the art, as the role of knockout mice in studying any of these conditions is both specific and substantial.

The Examiner argues that the phenotypes do not correlate with any disease or condition (page 5). The Examiner's arguments are similar to arguments made by the Patent Office with respect to pharmaceutical compounds the utility of which were based on murine model data, arguments which were dismissed by the Federal Circuit in *In re Brana* (34 U.S.P.Q.2d 1436)(Fed. Cir. 1995). The case involved compounds that were disclosed to be effective as anti-tumor agents and had demonstrated activity against murine lymphocytic leukemias implanted in mice. The court ruled that the PTO had improperly rejected, for lack of utility, claims for pharmaceutical compounds used in cancer treatment in humans, since neither the nature of

invention nor evidence proffered by the PTO would cause one of ordinary skill in art to reasonably doubt the asserted utility.

The first basis for the Board's holding of lack of utility (the Board adopted the examiner's reasoning without any additional independent analysis) was that the specification failed to describe any specific disease against which the claimed compounds were useful, and therefore, absent undue experimentation, one of ordinary skill in the art was precluded from using the invention. (*In re Brana* at 1439-40). The Federal Circuit reasoned that the leukemia cell lines were originally derived from lymphocytic leukemias in mice and therefore represented actual specific lymphocytic tumors. The court concluded that the mouse tumor models represented a specific disease against which the claimed compounds were alleged to be effective. (*In re Brana* at 1440).

The Board's second basis was that even if the specification did allege a specific use, the applicants failed to prove that the claimed compounds were useful.

The Federal Circuit responded: "[A] specification disclosure which contains a teaching of the manner and process of making and using the invention in terms which correspond in scope to those used in describing and defining the subject matter sought to be patented must be taken as in compliance with the enabling requirement of the first paragraph of Section 112 unless there is reason to doubt the objective truth of the statements contained therein which must be relied on for enabling support." (*Brana* at 1441, citing *In re Marzocchi*, 439 F.2d 220, 223, 169 USPQ 367, 369 (CCPA 1971)). From this it followed that the PTO has the initial burden of challenging a presumptively correct assertion of utility in the disclosure. Only after the PTO provides evidence showing that one of ordinary skill in the art would reasonably doubt the asserted utility does the burden shift to the applicant to provide rebuttal evidence sufficient to convince such a person of the invention's asserted utility. (*Id.*)

The court held that the Patent Office had not met its burden. The references cited by the Board did not question the usefulness of any compound as an antitumor agent or provide any other evidence to cause one of skill in the art to question the asserted utility of applicants' compounds. Rather, the references merely discussed the therapeutic predictive value of *in vivo* murine tests -- relevant only if the applicants were required to prove the ultimate value in humans of their asserted utility. The court did not find that the nature of the invention alone would cause one of skill in the art to reasonably doubt the asserted usefulness. The purpose of

treating cancer with chemical compounds did not suggest an inherently unbelievable undertaking or involve implausible scientific principles. (*Id.*)

The Court concluded that one skilled in the art would be without basis to reasonably doubt the asserted utility on its face. The PTO had not satisfied its initial burden. Accordingly, the applicants should not have been required to substantiate their presumptively correct disclosure to avoid a rejection under the first paragraph of Section 112. (*Id.*)

As in *Brana*, Applicant has asserted that the claimed invention is useful for a particular practical purpose, an assertion that would be considered credible by a person of ordinary skill in the art. As discussed above, the claimed mice have demonstrated specific phenotypes. The acceptance among those of skill in the art of knockout mice demonstrating such properties is clearly demonstrated.

Definitive proof that the phenotypes observed in the null mouse would be the same as those observed in humans is not a prerequisite to satisfying the utility requirement. It is enough that the claimed mouse demonstrates phenotypes, relative to a wild type control mouse, and that knockout mice are recognized in the art as models for determining gene function, both in mice and in humans. According to Austin et al.:

Among the model organisms, the mouse offers particular advantages for the study of human biology and disease: (i) the mouse is a mammal, and its development, body plan, physiology, behavior and diseases have much in common with those of humans; (ii) almost all (99%) mouse genes have homologs in humans; and (iii) the mouse genome supports targeted mutagenesis in specific genes by homologous recombination in embryonic stem (ES) cells, allowing genes to be altered efficiently and precisely.

(p. 921)(emphasis added).

In addition, as pointed out by Doetschman, one clearly skilled in the art, (*Laboratory Animal Science* 49:137-143, 137 (1999)(copy attached), the phenotypes observed in mice do correlate to gene function:

The conclusions will be that the knockout phenotypes do, in fact, provide accurate information concerning gene function, that we should let the unexpected phenotypes lead us to the specific cell, tissue, organ culture, and whole animal experiments that are relevant to the function of the genes in question, and that the absence of phenotype indicates that we have not discovered where or how to look for a phenotype.

(emphasis added).

In *Brana*, the claimed compound had demonstrated activity against a murine tumor implanted in a mouse. Yet, the Federal Circuit found that utility had been demonstrated. Here, the invention relates to a disruption in a murine gene in a mouse. Like the tumor mouse model, the knockout mouse with a specific gene disrupted is a widely accepted model, the utility of which would be readily accepted in the art. It is submitted that one skilled in the art would be without basis to be reasonably doubt Applicant's asserted utility, and therefore the Examiner has not satisfied the initial burden.

The Examiner states that the asserted use is not specific or substantial.

According to the MPEP, "specific utility" means "specific" to the subject matter claimed as compared to a "general utility" that would be applicable to the broad class of the invention (MPEP 2107.01). Use of the CASH $-/-$ mouse to study the function of the CASH gene and the association of the CASH gene with, for example, increased sensitivity to pain and increased susceptibility to seizure, is specific to this mouse. Even if there were many other genes associated with these phenotypes, only a CASH knockout mouse (as opposed to all other knockout mice) would be used to study the specific role of CASH in these phenotypes. The Examiner is respectfully requested to explain (1) how the asserted utility of determining the function of the CASH gene would be applicable to all other knockout mice; and (2) how the asserted use of studying the association of the CASH with pain and seizures would be applicable to all other knockout mice.

The Examiner asserts the claimed mice are not useful as research tools because using a product for further research is not a "substantial utility."

Applicant does not agree. First, it is wholly untrue that further research is required in order to confirm the utility of the claimed mouse in determining the function of CASH. The value of knockout mice in determining gene function is well established and accepted in the art. This is demonstrated by the references cited above. The Examiner has failed to provide sufficient factual support for the position that it is more likely than not that a person of skill in the art would doubt that Applicant's asserted utility is specific and substantial, which is the standard for establishing a *prima facie* case. See MPEP § 2107.02, IV.

Second, Applicant is claiming a transgenic mouse, and not the CASH or nucleic acid sequence. The Examiner must differentiate between the utility of the transgenic mouse and the utility of the target gene. "The claimed invention is the focus of the assessment of whether an

applicant has satisfied the utility requirement.” (MPEP 2107.02, I) That the claimed transgenic mouse can be used in a research setting to further characterize the CASH gene does not mean that the mouse lacks patentable utility. Further characterization (involving “basic research”) of the mouse itself is not necessary in order to confirm its utility in studying the function of the CASH gene.

The section entitled “Substantial Utility” provides:

A "substantial utility" defines a "real world" use. Utilities that require or constitute carrying out further research to identify or reasonably confirm a "real world" context of use are not substantial utilities. . . . the following are examples of situations that require or constitute carrying out further research to identify or reasonably confirm a "real world" context of use and, therefore, do not define "substantial utilities":

(A) Basic research such as studying the properties of the claimed product itself or the mechanisms in which the material is involved;

Office personnel must be careful not to interpret the phrase "immediate benefit to the public" or similar formulations in other cases to mean that products or services based on the claimed invention must be "currently available" to the public in order to satisfy the utility requirement. See, e.g., Brenner v. Manson, 383 U.S. 519, 534-35, 148 USPQ 689, 695 (1966). Rather, **any reasonable use that an applicant has identified for the invention that can be viewed as providing a public benefit should be accepted as sufficient, at least with regard to defining a "substantial" utility.**

(MPEP § 2107.01 I)(emphasis added).

The MPEP additionally provides:

Office personnel must distinguish between inventions that have a specifically identified substantial utility and inventions whose asserted utility requires further research to identify or reasonably confirm. **Labels such as** “research tool,” “intermediate” or **“for research purposes” are not helpful** in determining if an applicant has identified a specific and substantial utility for the invention.

(MPEP 2107.01, I)

A use is not substantial where further research is required to identify any use. This is not the case in the present application. Knockout mice have a well-known use in the study of gene function. In the present case, the instant invention does not require further research to establish a

utility. Applicant has determined that the CASH gene is associated with, for example, pain and seizures. No further research is required to establish any use. The invention has a “real world use” – as demonstrated by the delivery of the claimed invention to at least one large pharmaceutical company. Whether additional research is required to identify therapeutic agents targeting the CASH gene or to further characterize the function of the CASH gene is irrelevant to whether the claimed invention has satisfied the utility requirement.

The Examiner argues that Applicant did not state the purpose of the sale and that the specification does not disclose any such purpose.

Attached hereto is a Declaration from Robert Driscoll stating that knockout mice obtained from Deltagen are used for determining gene function and for drug discovery purposes – both uses which are clearly stated throughout the specification. It is apparent to one skilled in the art that the purpose of the sale of the CASH knockout mouse was to study the function of the CASH gene. To argue that the purpose of the sale is unknown is unreasonable, given that it is well established in the art that knockout mice are useful for studying gene function.

The Examiner argues that *Brana* does not apply.

Applicant submits that the legal principles as well as the facts of *Brana* are applicable to the present case. In *Brana*, the Board held that the applicant’s specification failed to disclose a specific disease against which the claimed compounds were useful. The Federal Circuit reversed and held that the mouse tumor model represented a specific disease against which the compounds were effective. In the present case, the Examiner has argued that Applicant failed to demonstrate a link between the CASH gene and any specific disease. It is Applicant’s position that a mouse demonstrating, for example, increased body fat is sufficient to establish the animal’s use as a model for metabolism disorders. Confirmation of the phenotype in humans is unnecessary.

As in the present case, in *Brana* the PTO did not consider the assertion of utility to be specific or credible:

Applicants’ specification, however, also states that the claimed compounds have “a better action and a better action spectrum as antitumor substances” than known compounds, specifically those analyzed in Paull. As previously noted, see supra note 4, Paull grouped various benzo [de]isoquinoline-1,3-diones, which had previously been tested in vivo for antitumor activity against two lymphocytic leukemia tumor models (P388 and L1210), into various structural classifications and analyzed the test results of the groups (i.e. what percent of the compounds in the particular group showed success

against the tumor models). Since one of the tested compounds, NSC 308847, was found to be highly effective against these two lymphocytic leukemia tumor models, 14 applicants' favorable comparison implicitly asserts that their claimed compounds are highly effective (i.e. useful) against lymphocytic leukemia. An alleged use against this particular type of cancer is much more specific than the vaguely intimated uses rejected by the courts in *Kirk* and *Kawai*. See, e.g., *Cross v. Iizuka*, 753 F.2d at 1048, 224 USPQ at 745 (finding the disclosed practical utility for the claimed compounds -- the inhibition of thromboxane synthetase in human or bovine platelet microsomes -- sufficiently specific to satisfy the threshold requirement in *Kirk* and *Kawai*.)

The Commissioner contends, however, that P388 and L1210 are not diseases since the only way an animal can get sick from P388 is by a direct injection of the cell line. The Commissioner therefore concludes that applicants' reference to Paull in their specification does not provide a specific disease against which the claimed compounds can be used. We disagree.

(*Brana* at 1440). Thus, the PTO was aware of the asserted use against the mouse tumor lines but did not find the use specific – as in the present case.

The court went on:

The ultimate issue is whether the Board correctly applied the Section 112 Para.1 enablement mandate and its implicit requirement of practical utility, or perhaps more accurately the underlying requirement of Section 101, to the facts of this case. As we have explained, the issue breaks down into two subsidiary issues: (1) whether a person of ordinary skill in the art would conclude that the applicants had sufficiently described particular diseases addressed by the invention, and (2) whether the Patent Act supports a requirement that makes human testing a prerequisite to patentability under the circumstances of this case.

The first subsidiary issue, whether the application adequately described particular diseases, calls for a judgment about what the various representations and discussions contained in the patent application's specification would say to a person of ordinary skill in the art. We have considered that question carefully, and, for the reasons we explained above in some detail, we conclude that the Board's judgment on this question was erroneous. Our conclusion rests on our understanding of what a person skilled in the art would gather from the various art cited, and from the statements in the application itself. We consider the Board's error to be sufficiently clear that it is reversible whether viewed as clear error or as resulting in an arbitrary and capricious decision.

The second subsidiary issue, whether human testing is a prerequisite to patentability, is a pure question of law: what does the practical utility requirement mean in a case of this kind. Under either our traditional standard or under the APA standard no deference is owed the Agency on a question of law, and none was accorded.

If the question concerning the standard of review, raised by the Commissioner, is to be addressed meaningfully, it must arise in a case in which the decision will turn on that question, and, recognizing this, the parties fully brief the issue. This is not that case.

We conclude that it is not necessary to the disposition of this case to address the question raised by the Commissioner; accordingly, we decline the invitation to do so.

(*Brana* at 1443-44). The court's position is reflected in the MPEP: if an "assertion would be considered credible by a person of ordinary skill in the art, do not impose a rejection based on lack of utility" (MPEP § 2107, II (A)(3); II (B)(1)). If it is well known to those skilled in the art that knockout mice are useful for studying gene function, then those skilled in the art would certainly regard such use as credible, specific and substantial. Nothing more is required to satisfy the statutory requirement. Applicant submits that, as in *Brana*, one skilled in the art would find the asserted use credible, substantial and specific.

The Examiner argues that the observed phenotypes cannot be reliably "contribute to disruption in CASH gene."

According to Example 4: "Heterozygous mice displayed a decrease in response time on the Hot Plate test. As shown in Figure 3, when compared to age-matched and gender-matched wild-type control mice, the heterozygous mutant mice responded significantly faster on the Hot Plate test, indicating that the heterozygous mice may have increased pain sensitivity."

According to Example 5: "The heterozygous mice displayed a decreased response threshold on the metrazol test. As shown in Figure 4, when compared to age-matched and gender-matched wild-type control mice, heterozygous mutant mice responded to a significantly lower dose on the metrazol test, indicating that these mice may be more seizure prone."

Applicant submits that these phenotypes were observed by comparing at least ten (10) F2N1 CASH +/- 129/OlaHsd x C57BL/6 mice with at least ten (10) age and gender matched wild-type F2N1 CASH ++ 129/OlaHsd x C57BL/6 mice - mice of identical background. This and additional information substantiating the findings set forth in the Examples is available in Deltagen's DeltaBase database which is licensed to Merck, Pfizer and GSK, among others. This information was available prior to the filing date of the present application.

Although the Applicant does not believe it necessary as a disclosure is presumed to be truthful, attached hereto is a Declaration executed by John Burke evidencing the above.

The Examiner is respectfully requested to explain how background could have caused the above reported phenotypes when the transgenic mice and the control mouse were of identical background. The reported results clearly support the Applicant's conclusion that the observed phenotypes were caused by the gene disruption.

The Examiner argues that the Applicant's "entire line of argument" is "misplaced and incorrect since utility analysis is for a particular claimed invention."

Contrary to the Examiner's statement, Applicant is not arguing a general utility for the claimed invention. Applicant is correctly applying the guidelines. According to the MPEP, "specific utility" means "specific" to the subject matter claimed as compared to a "general utility" that would be applicable to the broad class of the invention (MPEP 2107.01). It is Applicant's position that the claimed invention has a well-established use: to study gene function. It is Applicant's position that the use of any particular knockout mouse is specific to the study of that disrupted gene. In this case, the asserted use of the CASH knockout mouse is to study the function of the CASH gene – a specific use. As argued above, the Examiner is requested to explain how the asserted utility of determining the function of the CASH gene would be applicable to all other knockout mice – i.e., a general utility.

The Examiner further argues that the Applicant has not provided any substantial evidence that seizure or pain is specific to CASH gene disruption. The Examiner argues that pain and seizures are symptoms that occur in several gene disruptions or non-gene disruption conditions.

The Examiner is misconstruing the specific utility requirement set forth in the guidelines. The Examiner is implying that the term "specific" means "unique." The issue is not whether the phenotype is "specific" or "unique" to the CASH gene disruption but whether the use of the CASH mouse has any specific use which differentiates it from the broad class of invention (i.e. all other knockout mice). Clearly, many genes are involved in pain and seizures. However, the use of this claimed CASH transgenic mouse is specific to (1) the study of CASH gene function; and (2) to the study of the association of the CASH gene with pain and seizures. Thus, the asserted use is specific.

The Examiner has failed to respond to the separately asserted utility of gene expression analysis. As previously argued, the mice within the scope of claim 40 contain a *lacZ* gene. Their use in studying gene expression is clearly recognized by those skilled in the art:

Null-reporter alleles should be created

The project should generate alleles that are as uniform as possible, to allow efficient production and comparison of mouse phenotypes. The alleles should achieve a balance of utility, flexibility, throughput and cost. A null allele is an indispensable starting point for studying the function of every gene. Inserting a reporter gene (e.g., P-galactosidase or green fluorescent protein) allows a rapid assessment of which cell types normally support the expression of that gene.

(Austin et al., Nature Genetics (2004) 36(9):921-24, 922)(emphasis added)(copy attached). Applicant reminds Examiner that a claimed invention need only satisfy one of its stated objectives to satisfy the utility and enablement requirements.

As would be understood by one skilled in the art, the *lacZ* gene is inserted into the locus of the CASH allele. Expression is driven by the endogenous promoter. Expression of the *lacZ* gene indicates where the CASH gene is expressed. This use is specific for this mouse – knockout mice in general cannot be used for this purpose. The Examiner is requested to explain how this use would be applicable to all other knockout mice – i.e., a general utility.

In summary, Applicant submits that the claimed transgenic mouse, regardless of any disclosed phenotypes, has inherent and well-established utility in the study of the function of the gene, and thus satisfies the utility requirement of section 101. Moreover, Applicant believes that the transgenic mice are useful for studying CASH gene function with respect to the cited phenotypes, expression analysis, and are therefore useful for a specific practical purpose that would be readily understood by and considered credible by one of ordinary skill in the art.

In light of the arguments set forth above, Applicant does not believe that the Examiner has properly made a *prima facie* showing that establishes that it is more likely than not that a person of ordinary skill in the art would not consider that any utility asserted by the Applicant to be specific and substantial. (*In re Brana*; MPEP § 2107).

Rejections under 35 U.S.C. § 112, 1st paragraph

Claims 28-34 were rejected under 35 U.S.C. 112, first paragraph, for alleged lack of enablement. The Examiner contends that one skilled in the art would not know how to use the claimed invention, because the claimed invention is not supported by either a specific and substantial utility, or a well-established utility. Applicants traverse this rejection. For the reasons set forth above, the claimed invention satisfies the utility requirement. Therefore, one skilled in the art would know how to use the invention.

Withdrawal is respectfully requested.

Rejections under 35 U.S.C. § 112, 2nd paragraph

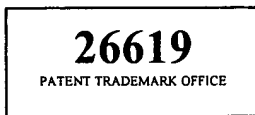
Claim 33 is rejected under 35 U.S.C. 112, second paragraph, as allegedly indefinite.

The claim has been amended to clarify the method steps. Withdrawal is respectfully requested.

Applicants submit that the claims are in condition for allowance and notification to that effect is earnestly solicited. The Examiner is invited to contact Applicants' representative if prosecution may be assisted thereby.

The Commissioner is hereby authorized to charge any deficiency or credit any overpayment to Deposit Account No. **502775**.

5-19-05
Date



Respectfully submitted,

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APPENDIX

Spencer, NHGRI; Background on Mouse as a Model Organism

Austin et al., Nature Genetics (2004) 36(9):921-24, 921, Commentary -
The Knockout Mouse Project

Doetschman, Laboratory Animal Science; April, 1999; 49(2):137-43,
Interpretation of Phenotype in Genetically Engineered Mice

Molecular Biology of the Cell (Albert, 4th ed., Garland Science (2002))

Genes VII (Lewin, Oxford University Press (2000))

Joyner (Gene Targeting: A Practical Approach, Oxford University Press 2000)

Maise et al. (Production of Targeted Embryonic Stem Cell Clones in Joyner,
Gene Targeting: A Practical Approach, Oxford University Press 2000)

Crawley (What's Wrong With My Mouse Behavioral Phenotyping of Transgenic
and Knockout Mice, Wiley-Liss 2000)



URL of this page: <http://www.genome.gov/10005834>

Background on Mouse as a Model Organism

Over the past century, the mouse has developed into the premier mammalian model system for genetic research. Scientists from a wide range of biomedical fields have gravitated to the mouse because of its close genetic and physiological similarities to humans, as well as the ease with which its genome can be manipulated and analyzed.

Although yeasts, worms and flies are excellent models for studying the cell cycle and many developmental processes, mice are far better tools for probing the immune, endocrine, nervous, cardiovascular, skeletal and other complex physiological systems that mammals share. Like humans and many other mammals, mice naturally develop diseases that affect these systems, including cancer, atherosclerosis, hypertension, diabetes, osteoporosis and glaucoma. In addition, certain diseases that afflict humans but normally do not strike mice, such as cystic fibrosis and Alzheimer's, can be induced by manipulating the mouse genome and environment. Adding to the mouse's appeal as a model for biomedical research is the animal's relatively low cost of maintenance and its ability to quickly multiply, reproducing as often as every nine weeks.

Mouse models currently available for genetic research include thousands of unique inbred strains and genetically engineered mutants. There are mice prone to different cancers, diabetes, obesity, blindness, Lou Gehrig's disease, Huntington's disease, anxiety, aggressive behavior, alcoholism and even drug addiction. Immunodeficient mice can also be used as hosts to grow both normal and diseased human tissue, a boon for cancer and AIDS research.

In the early days of biomedical research, scientists developed mouse models by selecting and breeding mice to produce offspring with the desired traits. Researchers also learned to produce useful, new models of genetic disease quickly and in large numbers by exposing mice to DNA-damaging chemicals, a process known as chemical mutagenesis.

In recent decades, researchers have utilized an array of innovative genetic technologies to produce custom-made mouse models for a wide array of specific diseases, as well as to study the function of targeted genes. One of the most important advances has been the ability to create transgenic mice, in which a new gene is inserted into the animal's germline. Even more powerful approaches, dependent on homologous recombination, have permitted the development of tools to "knock out" genes, which involves replacing existing genes with altered versions; or to "knock in" genes, which involves altering a mouse gene in its natural location. To preserve these extremely valuable strains of mice and to assist in the propagation of strains with poor reproduction, researchers have taken advantage of state-of-the-art reproductive technologies, including cryopreservation of embryos, *in vitro* fertilization and ovary transplantation.

The Jackson Laboratory, a publicly supported national repository for mouse models in Bar Harbor, Maine, has played a crucial role in the development of the mouse into the leading model for biomedical research. Established in 1929, the non-profit center pioneered the use of inbred laboratory mice to uncover the genetic basis of human development and disease. In fact, the famous "Black 6" or C57BL/6J mouse strain whose genome is the focus of the landmark sequencing effort was developed in the early 1920s by The Jackson Laboratory founder Clarence Cook Little.

Today, researchers at The Jackson Laboratory pursue projects in areas that include cancer, development and aging, immune system and blood disorders, neurological and sensory disorders, and metabolic diseases. Informatics researchers work with the public sequencing consortium to curate and integrate the sequenced mouse genome data with the wealth of biological knowledge collected in Jackson's Mouse Genome Informatics resource.

In addition, The Jackson Laboratory distributes 2,700 different strains and stocks as breeding mice, frozen embryos or DNA samples. In FY 2002 alone, the lab supplied approximately 2 million mice to the international scientific community.

Listed below is a sampling of mouse models developed and/or distributed by The Jackson Laboratory, along with brief descriptions of the human diseases they are helping scientists to understand:

- Down Syndrome - One of the most common genetic birth defects in humans, occurring once in every 800 to 1,000 live births, Down syndrome results from an extra copy of chromosome 21, an abnormality known as trisomy. The Ts65Dn mouse, developed at The Jackson Laboratory, mimics trisomy 21 and exhibits many of the behavioral, learning, and physiological defects associated with the syndrome in humans, including mental deficits, small size, obesity, hydrocephalus and thymic defects. This model represents the latest and best improvement of Down syndrome models to facilitate research into the human condition.

- Cystic Fibrosis (CF) - The *Cftr* knockout mouse has helped advance research into cystic fibrosis, the most common fatal genetic disease in the United States today, occurring in approximately one of every 3,300 live births. Scientists now know that CF is caused by a small defect in the gene that manufactures CFTR, a protein that regulates the passage of salts and water in and out of cells. Studies with the *Cftr* knockout have shown that the disease results from a failure to clear certain bacteria from the lung, which leads to mucus retention and subsequent lung disease. These mice have become models for developing new approaches to correct the CF defect and cure the disease.

- Cancer - The p53 knockout mouse has a disabled *Trp53* tumor suppressor gene that makes it highly susceptible to various cancers, including lymphomas and osteosarcomas. The mouse has emerged as an important model for human Li-Fraumeni syndrome, a form of familial breast cancer.

- Glaucoma - The DBA/2J mouse exhibits many of the symptoms that are often associated with human glaucoma, including elevated intraocular pressure. Glaucoma is a debilitating eye disease that is the second leading cause of blindness in the United States.

- Type 1 Diabetes - This autoimmune disease, also known as Juvenile Diabetes, or Insulin Dependent Diabetes Mellitus (IDDM), accounts for up to 10 percent of diabetes cases. Non-obese Diabetic (NOD) mice are enabling researchers to identify IDDM susceptibility genes and disease mechanisms.

- Type 2 Diabetes - A metabolic disorder also called Non-Insulin Dependent Diabetes Mellitus (NIDDM), this is the most common form of diabetes and occurs primarily after age 40. The leading mouse models for NIDDM and obesity research were all developed at The Jackson Laboratory: *Cpe^{fat}*, *Lep^{ob}*, *Lepr^{db}* and *tub*.

- Epilepsy - The "slow-wave epilepsy," or *swe*, mouse is the only model to exhibit both of the two major forms of epilepsy: petit mal (absence) and grand mal (convulsive). It shows particular promise for research into absence seizures, which occur most often in children.

- Heart Disease - Elevated blood cholesterol levels and plaque buildup in arteries within three months of birth (even on a low-fat diet) are characteristics of several experimental models for

human atherosclerosis: the *ApoE* knockout mouse and C57BL/6J.

- Muscular Dystrophy - The *Dmd*^{mdx} mouse is a model for Duchenne Muscular Dystrophy, a rare neuromuscular disorder in young males that is inherited as an X-linked recessive trait and results in progressive muscle degeneration.

- Ovarian Tumors - The SWR and SWXJ mouse models provide excellent research platforms for studying the genetic basis of ovarian granulosa cell tumors, a common and very serious form of malignant ovarian tumor in young girls and post-menopausal women.

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The Knockout Mouse Project

Mouse knockout technology provides a powerful means of elucidating gene function *in vivo*, and a publicly available genome-wide collection of mouse knockouts would be significantly enabling for biomedical discovery. To date, published knockouts exist for only about 10% of mouse genes. Furthermore, many of these are limited in utility because they have not been made or phenotyped in standardized ways, and many are not freely available to researchers. It is time to harness new technologies and efficiencies of production to mount a high-throughput international effort to produce and phenotype knockouts for all mouse genes, and place these resources into the public domain.

Now that the human and mouse genome sequences are known¹⁻³, attention has turned to elucidating gene function and identifying gene products that might have therapeutic value. The laboratory mouse (*Mus musculus*) has had a prominent role in the study of human disease mechanisms throughout the rich, 100-year history of classical mouse genetics, exemplified by the lessons learned from naturally occurring mutants such as agouti⁴, reder⁵ and obese⁶. The large-scale production and analysis of induced genetic mutations in worms, flies, zebrafish and mice have greatly accelerated the understanding of gene function in these organisms. Among the model organisms, the mouse offers particular advantages for the study of human biology and disease: (i) the mouse is a mammal, and its development, body plan, physiology, behavior and diseases have much in common with those of humans; (ii) almost all (99%) mouse genes have homologs in humans; and (iii) the mouse genome supports targeted mutagenesis in specific genes by homologous recombination in embryonic stem (ES) cells, allowing genes to be altered efficiently and precisely.

The ability to disrupt, or knock out, a specific gene in ES cells and mice was developed in the late 1980s (ref. 7), and the use of knockout mice has led to many insights into human biology and disease⁸⁻¹¹. Current technology also permits insertion of 'reporter' genes into the knocked-out gene, which can then be used to determine the temporal and spatial

expression pattern of the knocked-out gene in mouse tissues. Such marking of cells by a reporter gene facilitates the identification of new cell types according to their gene expression patterns and allows further characterization of marked tissues and single cells.

Appreciation of the power of mouse genetics to inform the study of mammalian physiology and disease, coupled with the advent of the mouse genome sequence and the ease of producing mutated alleles, has catalyzed public and private sector initiatives to produce mouse mutants on a large scale, with the goal of eventually knocking out a substantial portion of the mouse genome^{12,13}. Large-scale, publicly funded gene-trap programs have been initiated in several countries, with the International Gene Trap Consortium coordinating certain efforts and resources¹⁴⁻¹⁷.

Despite these efforts, the total number of knockout mice described in the literature is relatively modest, corresponding to only ~10% of the ~25,000 mouse genes. The curated Mouse Knockout & Mutation Database lists 2,669 unique genes (C. Rathbone, personal communication), the curated Mouse Genome Database lists 2,847 unique genes, and an analysis at Lexicon Genetics identified 2,492 unique genes (B.Z., unpublished data). Most of these knockouts are not readily available to scientists who may want to use them in their research; for example, only 415 unique genes are represented as targeted mutations in the Jackson Laboratory's Induced Mutant Resource database (S. Rockwood, personal communication).

The converging interests of multiple members of the genomics community led to a meeting to discuss the advisability and feasibility of

a dedicated project to produce knockout alleles for all mouse genes and place them into the public domain. The meeting took place from 30 September to 1 October 2003 at the Banbury Conference Center at Cold Spring Harbor Laboratory. The attendees of the meeting are the authors of this paper.

Is a systematic project warranted?

A coordinated project to systematically knock out all mouse genes is likely to be of enormous benefit to the research community, given the demonstrated power of knockout mice to elucidate gene function, the frequency of unpredicted phenotypes in knockout mice, the potential economies of scale in an organized and carefully planned project, and the high cost and lack of availability of knockout mice being made in current efforts. Moreover, implementing such a systematic and comprehensive plan will greatly accelerate the translation of genome sequences into biological insights. Knockout ES cells and mice currently available from the public and private sectors should be incorporated into the genome-wide initiative as much as possible, although some may be needed to be produced again if they were made with suboptimal methods (e.g., not including a marker) or if their use is restricted by intellectual property or other constraints. The advantages of such a systematic and coordinated effort include efficient production with reduced costs; uniform use of knockout methods, allowing for more comparability between knockout mice; and ready access to mice, their derivatives and data to all researchers without encumbrance. Solutions to the logistical, organizational and informatics issues associated with producing, characterizing and distributing such a large number of

*The Comprehensive Knockout Mouse Project Consortium**

*Authors and their affiliations are listed at the end of this paper.



COMMENTARY

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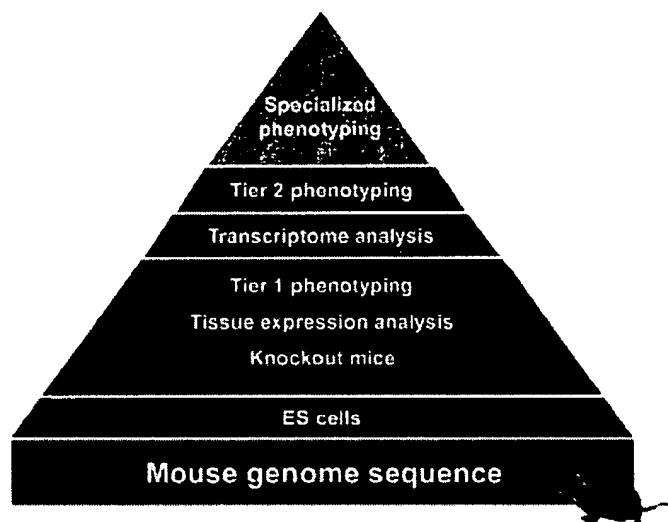


Figure 1 Structure of resource production in the proposed KOMP. Using the mouse genome sequence as a foundation, knockout alleles in ES cells will be produced for all genes. A subset of ES cell knockouts will be used each year to produce knockout mice, determine the expression pattern of the targeted gene in a variety of tissues and carry out screening-level (Tier 1) phenotyping. In a subset of mouse lines, transcriptome analysis and more detailed system-specific (Tier 2) phenotyping will be done. Finally, specialized phenotyping will be done on a smaller number of mouse lines with particularly interesting phenotypes. All stages will occur within the purview of the KOMP except for the specialized phenotyping, which will occur in individual laboratories with particular expertise.

mice will draw from the experience of related projects in the private sector and in academia, which have made or phenotyped hundreds of knockout mice using a variety of techniques. Lessons learned from these projects include the need for redundancy at each step to mitigate pipeline bottlenecks and the need for robust informatics systems to track the production, analysis, maintenance and distribution of thousands of targeting constructs, ES cells and mice.

Null-reporter alleles should be created
The project should generate alleles that are as uniform as possible, to allow efficient production and comparison of mouse phenotypes. The alleles should achieve a balance of utility, flexibility, throughput and cost. A null allele is an indispensable starting point for studying the function of every gene. Inserting a reporter gene (e.g., β -galactosidase or green fluorescent protein) allows a rapid assessment of which cell types normally support the expression of that gene. Therefore, we propose to produce a null-reporter allele for each gene. Making each mutation conditional in nature by adding *cis*-elements (e.g., *loxP* or FRT sites) would

be desirable, but we do not advocate this as part of the mutagenesis strategy unless the technological limitations currently associated with generating conditional targeted mutations on a large scale and in a cost-effective manner can be overcome.

A combination of methods should be used. Various methods can be used to create mutated alleles, including gene targeting, gene trapping and RNA interference. Advantages of conventional gene targeting include flexibility in design of alleles, lack of limitation to integration hot spots, reliability for producing complete loss-of-function alleles, ability to produce reporter knock-ins and conditional alleles, and ability to target splice variants and alternative promoters. BAC-based targeting has the potential advantages of higher recombination efficiencies and flexibility for producing complex mutated alleles¹⁸. Gene trapping is rapid, is cost-effective and produces a large variety of insertional mutations throughout the genome but can be somewhat less flexible^{17,19–21}. There is uncertainty regarding the percentage of gene traps that produce a true null allele and the fraction

of the genome that can ultimately be covered by gene-trap mutations. Trapping is not entirely random but shows preference for larger transcription units and genes more highly expressed in ES cells. In recent studies, gene trapping was estimated to potentially produce null alleles for 50–60% of all genes, perhaps more if a variety of gene-trap vectors with different insertion characteristics is used^{17,21}. RNA interference offers enormous promise for analysis of gene function in mice²² but is not yet sufficiently developed for large-scale production of gene modifications capable of reliably producing true null alleles. Both gene-targeting and gene-trapping methods are suitable for producing large numbers of knockout alleles, and, given their complementary advantages, a combination of these methods should be used to produce the genome-wide collection of null-reporter alleles most efficiently.

What should the deliverables be?

A genome-wide knockout mouse project could deliver to the research community a trove of valuable reagents and data, including targeting and trapping constructs and vectors, mutant ES cell lines, live mice, frozen sperm, frozen embryos, phenotypic data at a variety of levels and detail, and a database with data visualization and mining tools. At a minimum, we believe that a comprehensive genome-wide resource of mutant ES cell lines from an inbred strain, each with a different gene knocked out, should be produced and made available to the community. Choosing an inbred line (129/SvEvTac or C57BL/6J), and evaluating the alternative of using F₁ ES cells and tetraploid aggregation to provide potential time savings, merits additional scientific review and discussion^{23,24}. ES cells should be converted into mice at a rate consistent with project funding and the ability of the worldwide scientific community to analyze them. Although the value and cost-effectiveness of systematically characterizing the mice is a matter of debate, a limited set of broad and cost-effective screens, probably including assessment of developmental lethality, physical examination, basic blood tests, and histochemical analysis of reporter gene expression, would be useful. More detailed phenotyping, based on findings from the initial screen or existing knowledge of the gene's function, could be done at specialized centers. All ES cell clones and mice (as frozen embryos or sperm) should be available to any researcher at minimal cost, and all mouse phenotyping and reporter expression data should be deposited into a public database.

In determining how to implement the project, utility to the research community should be the standard for judging value. Each step after ES cell generation (e.g., mouse creation, breeding, expression analysis, phenotyping) will make the resource useful to more researchers but will also increase costs and scientific complexity. We therefore advocate a 'pyramid' structure for the project (Fig. 1). At the base of the pyramid is the genome-wide collection of mutant ES cells for every mouse gene. Over time, a subset of these mutant ES cells should be made into mice and characterized with an initial phenotype screen (Tier 1; Fig. 1) and analysis of tissue reporter-gene expression. A subset of these lines should be profiled by microarray analysis, and a subset of these profiled by system-specific (Tier 2) phenotyping, based on the results of the Tier 1 phenotyping, array studies, existing knowledge of the gene's function and the gene's tissue expression pattern. With time, the upper tiers of the pyramid will be filled out, eventually transforming the pyramid into a cube, with information of all types available for all genes.

This project will require the resolution of numerous intellectual property claims involving the production and use of knockout mice. To deal with the existing patents that cover the technologies and processes involved in the production of mutant mice, we suggest that a 'patent pool', such as that used in the semiconductor industry²⁵, should be generated. Several individuals who represent entities that control patents on mouse knockout technologies are authors on this paper, and they agree with this approach. We also agree that any mutant ES cells or mice produced should be placed immediately in the public domain.

Mechanisms and costs

ES cell production. Automated knockout construct and ES cell production should be carried out in coordinated centers to ensure efficiency and uniformity. We estimate that most known mouse genes could be knocked out in ES cells within 5 years, using a combination of gene-trapping and gene-targeting techniques. Gene trapping can produce a large number of mutated alleles quickly, but its progress should be monitored closely to determine when its yield of new genes diminishes¹⁷ and, therefore, when targeting should be increasingly relied on. As large-scale trapping projects have already defined gene classes that probably cannot be knocked out by trapping (e.g., single-exon GPCRs, genes that are not expressed in ES cells), we propose that targeting begin on those classes immediately. All ES cells should be made available to the research community, because this collection itself

would be a valuable resource. Efforts in the public and private sectors have already knocked out many genes in ES cells, and, to the degree that the alleles produced fit the prescribed characteristics (i.e., null alleles with a reporter) and are available, every effort should be made to incorporate these into the planned public resource. Costs for generating this part of the resource were estimated at between \$9–11 million/year for five years (these and all subsequent figures are direct costs).

Mouse production. The subset of ES cells made into mice each year should be chosen by a peer-review process. Central facilities for high-efficiency mouse production, genotyping, breeding, maintenance and archiving should be funded, to take advantage of efficiencies of scale in mouse creation and distribution. Researchers could apply to produce groups of mice outside the centers, as long as they meet the cost specifications of the program. All mice should be made available immediately to researchers as frozen embryos or sperm, for nominal distribution cost. An initial target of 500 new mouse lines per year would double the current rate at which new genes are knocked out in the public sector; we feel that this rate is within the capacity of the biomedical research community worldwide to absorb and analyze. We estimated the initial cost of this level of mouse production to be \$12.5–15 million per year.

Reporter tissue expression analysis. Approximately 30 tissues from adult and developmental stages should be sampled to cover the main organ systems. Analysis methods should be customized to the organ system and marker, and a searchable database of the sites of gene expression, and the images showing them, should be produced. Centers to carry out these analyses and data curation should be selected by peer review. We estimated the cost of this component for 500 mouse lines to be \$2.5–5 million per year, depending on how much tissue sectioning and cell-level analysis is done.

Phenotyping. Tier 1 phenotyping should be a low-cost screen for clear phenotypes and should be done on all mouse lines produced. Tier 1 should include home-cage observation, physical examination, blood hematological and chemistry profiles, and skeletal radiographs. The centers producing the mice should carry out the Tier 1 analyses, at an estimated cost of \$2.5 million per year for 500 lines. Selected lines, chosen on the basis of findings from Tier 1 phenotyping, tissue expression patterns, microarray data and the scientific literature, should undergo more detailed and system-focused Tier 2 phenotyping. Tier 2 phenotyping should be done in

specialized phenotyping centers, akin to those already in operation for phenotyping of mice produced by ENU mutagenesis. All Tier 1 and Tier 2 phenotyping should be done on a uniform genetic background by dedicated groups of individuals in single locations, to facilitate consistency and cross-comparison of results among different mouse lines. All Tier 1 and Tier 2 phenotyping results should be deposited into a central project database freely accessible to the research community. More detailed and specialized phenotyping could be done by individual researchers in their own laboratories; deposition of this more detailed phenotype data would be encouraged.

Transcriptome analysis. Transcriptome profiling of tissues from each knockout line, collected in a uniform way across all mice and tissues and placed into a searchable relational database, would add substantially to the scientific value of the project, though it would also add considerably to its cost. Transcriptome analysis should therefore be done on a subset of mice, chosen by peer review. We estimate that, with the best currently available array technology, an analysis of ten tissues would cost ~\$18,000 per line.

Conclusions

This project, tentatively named the Knockout Mouse Project (KOMP), will be a crucial step in harnessing the power of the genome to drive biomedical discovery. By creating a publicly available resource of knockout mice and phenotypic data, KOMP will knock down barriers for biologists to use mouse genetics in their research. The scientific consensus that we achieved—that a dedicated project should be undertaken to produce mutant mice for all genes and place them into the public domain—is important but is only the beginning. Implementation of these recommendations will require additional input from the greater scientific community, including those responsible for programmatic direction and financial support of biomedical research in the public and private sectors. This ambitious and historic initiative must be carried out as a collaborative effort of the worldwide scientific community, so that all can contribute their skills, and all can benefit. International discussions among scientific and programmatic staffs since the Banbury meeting at Cold Spring Harbor, in both the public and private sectors, have shown that there is great enthusiasm and commitment to this vision. The next step for KOMP will be to move this visionary plan from conceptualization to implementation, with an urgency befitting the benefits it will bring to science and medicine.

COMMENTARY

URLs. The curated Mouse Knockout & Mutation Database is available at <http://research.bmn.com/mkmd/>. The curated Mouse Genome Database is available at <http://www.informatics.jax.org/>. Patent pools: A solution to the problem of access in biotechnology patents? is available at <http://www.uspto.gov/web/offices/pac/dapp/opla/patentpool.pdf>.

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Special Topic Overview

Interpretation of Phenotype in Genetically Engineered Mice

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Background and Purpose: In mice, genetic engineering involves two general approaches—addition of an exogenous gene, resulting in transgenic mice, and use of knockout mice, which have a targeted mutation of an endogenous gene. The advantages of these approaches is that questions can be asked about the function of a particular gene in a living mammalian organism, taking into account interactions among cells, tissues, and organs under normal, disease, injury, and stress situations.

Methods: Review of the literature concentrating principally on knockout mice and questions of unexpected phenotypes, lack of phenotype, redundancy, and effect of genetic background on phenotype will be discussed.

Conclusion: There is little gene redundancy in mammals; knockout phenotypes exist even if none are immediately apparent; and investigating phenotypes in colonies of mixed genetic background may reveal not only more phenotypes, but also may lead to better understanding of the molecular or cellular mechanism underlying the phenotype and to discovery of modifier gene(s).

One often hears the comment that genetically engineered mice, especially knockout mice, are not useful because they frequently do not yield the expected phenotype, or they don't seem to have any phenotype. These expectations are often based on years of work, and in some instances, thousands of publications of mostly in vitro studies. Examples of unexpected phenotypes, based largely on experience with transforming growth factor beta (*Tgfb*) and basic fibroblast growth factor (*Fgf2*) knockout and transgenic mice, will be presented to discuss possible reasons for unexpected knockout phenotypes. The conclusions will be that the knockout phenotypes do, in fact, provide accurate information concerning gene function, that we should let the unexpected phenotypes lead us to the specific cell, tissue, organ culture, and whole animal experiments that are relevant to the function of the genes in question, and that the absence of phenotype indicates that we have not discovered where or how to look for a phenotype.

Before entering into how one should interpret unexpected knockout phenotypes and how one should deal with lack of knockout phenotypes, it is necessary to give a brief introduction into how knockout mice are made. For detailed information, the following reviews are suggested (1–4). Transgenic technology has had a long history; thus, an introduction to that technology will not be given here. Rather, the following reviews are suggested (5, 6). At this juncture, it should be noted that, although transgenic vertebrates ranging from fish to bovines have been produced, knockout technology has

to date been successful only in mice, even though embryonic stem (ES) cells have been produced from several other species, including hamster (7), rat (8), rabbit (9, 10), pig (11–13), bovine (14, 15), and zebrafish (16). Consequently, the entire discussion will be focused on mice.

Knockout mice are generated by the injection of genetically engineered or gene-targeted ES cells into a mouse blastocyst to generate a chimeric embryo, which in turn can pass on the engineered gene to its offspring. ES cell lines are established from the inner cell mass of a mouse blastocyst, so that when injected into blastocysts, the ES cells can incorporate into the inner cell mass of the recipient blastocysts thereby chimerizing them. Subsequent to transfer of the chimeric blastocysts into uteri of pseudopregnant mice, chimeric mice are born. If the germline of a chimeric mouse is colonized by cells derived from the injected ES cells, the chimera is termed a "germline" chimera. Some of the offspring of the germline chimeras will then carry the engineered gene in their genomes. Gene targeting in ES cells uses the ES cells' DNA repair apparatus to bring about homologous recombination between an exogenous DNA fragment transfected into the ES cell and its homologous region in the genome. Homologous recombination usually results in replacement of the endogenous region with the exogenous fragment, thereby altering the endogenous gene in a prespecified manner. There are many variations on this procedure by which genes can be altered not only to ablate function, but also to make more subtle mutations (17–19). Such procedures can be used to introduce point mutations, remove specific splicing products, switch isoforms, and humanize genes. In addition, technology has recently been

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developed to make conditional and inducible knockouts in which gene function is ablated either in a developmentally specified tissue (20–22) or in an inducible manner (23–26). These techniques, though exciting, will not be further discussed.

Extensive nonredundancy in the TGF β family: Several thousand cell culture studies on the three mammalian transforming growth factor beta proteins (TGF β s 1, 2, and 3) have implicated these growth and differentiation factors in the function of nearly every cell type studied. Expression studies indicated unique and overlapping expression of the three TGF β s (27, 28). For example, overlapping protein localization was found in all gut epithelia, all layers of the skin, all three muscle types, kidney tubules, lung bronchi, cartilage, and bone (Table 1). Together with the fact that all three TGF β s signal through a common TGF type-II receptor (Figure 1), these data strongly suggest considerable redundancy in function. Consequently, it is surprising that, of the >30 phenotypes of the three *Tgfb* knockout mice that we have described (29–31), none appear to be overlapping (Table 2). These results indicate extensive nonredundancy between TGF β ligands even though there is considerable overlap in expression. Of course, these results do not rule out the possibility of some redundancy in some tissues. Combination of the ligand knockouts would uncover such situations, and it is likely that a few will exist, but 30 non-overlapping phenotypes for three ligands strongly suggests that a vast number of their functions are not redundant.

There are several possible explanations for how there can be so much overlap in ligand expression and yet so much specific ligand function. First, TGF β s are secreted as latent peptides and must be activated before they can bind receptors (32–35). The mechanism by which this extracellular processing occurs is not well understood and may be different for each TGF β . Hence, ligand processing presumably determines some functional specificity for the three TGF β s. Second, there is a third type of TGF β receptor, TGF β R3, that can interact with ligand and receptor types I and II before cytoplasmic signaling can occur, though involvement of TGF β R3 is not essential for signaling (36–38). Association with type III receptors is thought to enhance some TGF β R1 and 2/ligand interactions. Upon ligand binding, the serine/threonine receptor TGF β R2 then associates with and phosphorylates the transmembrane serine/threonine receptor TGF β R1, which in turn initiates a phosphorylation-mediated signaling cascade. Hence, combinatorial receptor/ligand interactions will also determine functional specificity. Third, signaling from TGF β R1 can occur through two cytoplasmic signaling proteins called SMAD2 and 3 (39, 40) and, perhaps, through a third called SMAD5 (41). In addition, SMAD6 and 7 can also interact with the other SMADs to inhibit signaling (42–44). Hence, differential SMAD protein interactions with transcriptional machinery will probably also determine functional specificity for the three TGF β ligands. Finally, there may be several non-transcriptional signaling pathways for TGF β s. For example, we have found that TGF β 1-deficient platelets from *Tgfb1* knockout mice have impaired platelet aggregation that can be restored by incubating isolated platelets with recombinant TGF β 1 (unpublished observations). Because platelets do not have a

Table 1. Protein expression of transforming growth factor beta (TGF β) 1, 2, and 3

Tissue/cell type	TGF β 1	TGF β 2	TGF β 3
Cartilage			
Perichondrium	+++	+	++
Chondrocytes	+	++	++
Bone			
Periosteum	++	-	+
Osteocytes		++	++ ++
Tooth			
Ameloblasts	+	-	+
Odontoblasts	-	++	-
Pulp	+	+++	+
Muscle			
Smooth	+	+	++
Cardiac	+	+	+++
Skeletal	+	++	
Lung			
Bronchi	++	++	++
Alveoli	-	-	-
Blood vessels			
Endothelium	-	-	++
Smooth muscle	+	+	+++
Kidney			
Tubules	++	++	++
Basement membrane	-	+++	-
Adrenal			
Cortex	+++	+++	-
Medulla	-	-	-
Gut			
Esophageal epithelium	+++	+	+
Gastric epithelium	+++	+	+
Intestinal epithelium	++	+	+
Basement membrane	-	+++	-
Muscularis	+	+	++
Liver			
Capsule	-	-	++
Parenchyma	-	-	-
Megakaryocytes	+	-	++
Eye			
Lens epithelium	-	-	-
Lens fibers	+++	+	+
Ear			
Cochlear epithelium	-	+	+++
Basement membrane	-	+++	-
CNS			
Meninges	+	+++	+
Glial	-	++	++
Choroid plexus	-	-	++
Skin			
Periderm	++	+	++
Epidermis	+++	+++	+++
Dermis	+	+++	++
Hair follicles	++	++	+

The polyclonal antibodies used were specific for residues 4–19 of TGF β 1 and 2 and residues 9–20 of TGF β 3. The avidin-biotin system was used for staining. Data obtained from immunohistochemical study of Pelton et al. (28). Reproduced from *The Journal of Cell Biology*, 1991,115:1091–1105, by copyright permission of The Rockefeller University Press.

nucleus, there must exist a signaling pathway that is nontranscriptional. In summary, given the complexities of ligand processing, receptor interactions, and signaling pathways, it becomes clear why redundancy in TGF1, 2, and 3 function has not been detected at the whole animal level, even though there is considerable overlap in expression of *Tgfb* gene family members. Consequently, if other gene families function with similar complexity, it is likely that, in the final analysis, little functional redundancy will be found within gene families.

Two striking examples of apparent functional redundancy are worth considering. The first involves myogenic genes, and the second involves retinoic acid receptors. Contrary to early interpretations, redundancy does not now appear to be

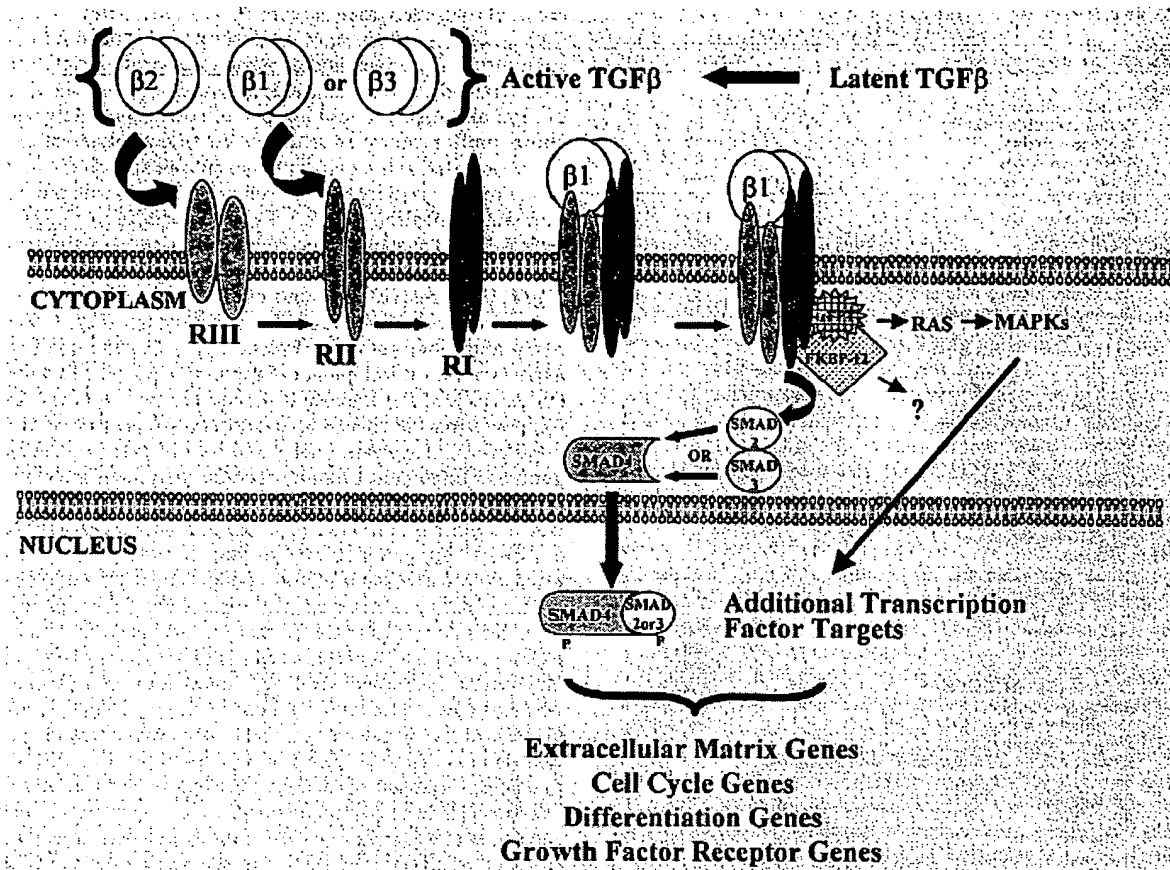


Figure 1. TGFβ signaling pathway. The TGFβ ligands, TGFβ1 (β1), TGFβ2 (β2), and TGFβ3 (β3), exist primarily in a latent form in vivo and are activated by mechanisms not yet clear. In general, TGFβ2 interacts with a TGFβ type III receptor (RIII) before interaction with TGFβ type II (RII) and TGFβ type I (RI) receptors; whereas, the TGFβ1 and TGFβ3 ligands can interact directly with the type II receptor. The ligand receptor complexes can then associate with several cytoplasmic molecules, farnesyl protein transferase (FPT) and FK506 binding protein-12 (FKBP-12), being two potential examples. The receptor-ligand complex signals to the nucleus through threonine/serine phosphorylation of a series of SMAD proteins (related to the *Drosophila* "mothers against decapentaplegic" protein) which then elicit transcriptional regulation of extracellular matrix, cell cycle, differentiation and growth factor receptor genes. The roles of the associated cytoplasmic molecules FPT and FKBP-12 are not clear but are thought to involve RAS pathway signaling and modulation of signaling through the SMAD proteins.

the case for two of the myogenic genes known to be essential for specification of vertebrate skeletal muscle, *MyoD* and *Myf5*. Even though the individual knockouts have muscle, and only the combined knockouts do not have muscle (45), it is now clear that each gene functions in the specification of distinct muscle cell lineages. Consequently, in the absence of one source of muscle cells, the other source may compensate for that (46, 47). This should be termed developmental compensation, rather than gene redundancy. On the other hand, with respect to retinoic acid receptors, there is also good evidence for functional redundancy. Similar to the myogenic genes, retinoic acid receptor gene knockout mice have few phenotypes, whereas the combined knockouts have many phenotypes (48, 49). Whether this turns out to be gene redundancy or another case of developmental compensation remains to be determined.

Lack of phenotype: As is the case for TGFβ, there also is a multitude of reports indicating that the FGFs 1 and 2 have important roles in numerous cell types and tissues. Consequently, when the *Fgf2* gene was knocked out by gene targeting, it was quite surprising that there was no obvious phenotype (50). The *Fgf2*^{-/-} animals live a long, healthy life, and fertility and fecundity are normal. Even the pituitary gland, which is the best source of FGF2, appears not to have morphologic defects. The only evidence for any developmental abnormalities is found in hematopoiesis (50), where blood platelet counts are high, and in the cerebral cortex (51, 52), where morphometric analysis reveals decreased cell density. Clearly, these abnormalities are minor, compared with expectations. This was all the more evident because our transgenic mice, in which the human *FGF2* gene was ubiquitously overexpressed by the phosphoglycerate kinase pro-

Table 2. Nonoverlapping phenotypes of *Tgfb1*, 2, and 3 knockout mice and the penetrance of those phenotypes

Knockout mouse phenotype	Penetrance (%) ^a
<i>Tgfb1</i>	
Embryo lethality	50
Preimplantation lethality	50
Yolk sac lethality	50 ^b
Adult phenotypes	50
Multifocal autoimmunity	100 ^c
Platelet defect	100 ^d
Colon cancer	100 ^d
Failing heart	100 ^d
<i>Tgfb2</i> (all perinatal lethality)	
Heart defects	100
Ventricular septum defects	94
Dual outlet right ventricle	19
Dual inlet left ventricle	25
Inner ear defect—lacks spiral limbus	100
Eyes	
Ocular hypercellularity	100
Reduced corneal stroma	100
Urogenital defects in kidney	
Dilated renal pelvis	30
Agensis (females only)	20
Uterine horn ectopia	40
Testicular ectopia	100
Testis hypoplasia	20
Vas deferens dysgenesis	20
Lung-postnatal	
Dilated conducting airways	100
Collapsed bronchioles	100
Skeletal defects	
Occipital bone	100
Parietal bone	100
Squamous bone	100
Palatine bone (cleft palate)	22
Alisphenoid bone	100
Mandibular defects	100
Short radius and ulna	100
Missing deltoid tuberosity and third trochanter	94
Sternum malformations	25
Rib barreling	94
Rib fusions	13
Spina bifida	100
<i>Tgfb3</i> (perinatal lethality)	
Cleft palate	100

^aSee Table 3 for background dependency of *Tgfb1* knockout phenotypes.

^bDescribed in references 64, 67.

^cRefers to percentage penetrance among animals that survive to birth.

^dUnpublished observations.

Details on the remaining phenotypes can be found in the text and in references 29–31, 63.

moter (53), had very short legs, suggesting an important role of FGF2 in bone development, yet the bones of the knockout animals were normal. This apparent discrepancy between the transgenic and knockout mice indicates that some other FGF signals through the same FGF receptor as does FGF2, and that this other FGF is the true ligand that is important in bone development. Another possibility is that there is "developmental compensation" by alternative mechanisms. In other words, the absence of FGF2 may cause developmental abnormalities during bone development that are then compensated for by another developmental pathway. This alternative would not necessarily require a different FGF to be involved.

After we had made our first analysis of the *Fgf2* knockout mouse and did not find an obvious phenotype, it was easy to explain the "lack of phenotype" by invoking redundancy because there are at least 18 known *Fgf* genes. But in hindsight, it now appears more likely that all members of this large gene family have specific functions, even though they

signal through receptors encoded by only four receptor genes (54). In *Fgf2* knockout mice, evidence was not found for up-regulation of the two ligands most structurally related to FGF2, namely, FGFs 1 and 5 (50). Also, genetic combination of *Fgf2* and *Fgf5* (50) did not reveal redundancy between these similar genes. In addition, further analysis of the mice revealed roles being played in hematopoiesis and vascular tone control (50) as well as in brain development and wound healing (51, 52). Finally, in addition to *Fgf2*, *Fgfs* 3–5, 7, 8 also have been ablated by gene targeting, revealing functions in proliferation of the inner cell mass (*Fgf4*) (55); gastrulation and cardiac, craniofacial, forebrain, midbrain, and cerebellar development (*Fgf8*) (56); brain and inner ear development (*Fgf3*) (57, 58); and two aspects of hair development (*Fgf5* and 7) (59, 60). To date, comparison of *Fgf* knockout phenotypes from 6 of the 18 *Fgf* genes has not turned up overlap except possibly in the cerebellum. Together, these results indicate that each gene has important unique functions. Although a few redundant functions may eventually be found on combination of *Fgf2* with all other *Fgfs* except *Fgf5*, it is clear that 6 of the 18 *Fgf* genes studied by gene targeting have been associated with essentially unique knockout phenotypes.

To summarize, what originally appeared as "lack of phenotype" led many of us to the premature conclusion that other FGFs must have functions redundant to those of FGF2. However, further analysis of *Fgf2* knockout mice has since revealed a wealth of unique functions ranging from thrombocytosis and vascular tone control (50) to brain development and wound healing (51, 52). It is my expectation that further physiologic analysis of the *Fgf2* knockout mouse will reveal functions in the hypertrophic response to hypertension and responses to ischemia/reperfusion injury and bone injury. In the final analysis, it is likely that the major roles of FGF2 may have less to do with getting us to birth than with keeping us alive after birth, whereas several other FGFs clearly have developmental roles.

Effects of genetic background on phenotypic variation: From 100 years of mouse genetics, it has become clear that genetic background plays an important role in the susceptibility of mice to many disorders. Therefore, the phenotypes of knockout mouse strains will also have genetic background dependencies, as was first documented by the Magnuson and Wagner groups (61, 62). The *Tgfb1* knockout mice are an exceptional case in point (Table 3). On a mixed (50:50) 129 x CF1 background (CF1 is a partially outbred strain), about half of *Tgfb1* knockout mice die from a preimplantation developmental defect (63), and the other half die of an autoimmune-like multifocal inflammatory disease at about weaning age (29). If the targeted *Tgfb1* allele is backcrossed onto a C57BL/6 background, 99% of all knockout animals die of the preimplantation defect (63). However, if a *Tgfb1* knockout allele is put onto a mixed 129 x NIH/Ola x C57BL/6 background, embryo lethality is observed during yolk sac development, not during preimplantation development (64). With respect to the multifocal inflammatory disorder of *Tgfb1* knockout mice, if the targeted allele is put onto a 129 x CF1 mixed background (50:50), severe inflammation exists only in the stomach (29); on the mixed 129 x

Table 3. Background dependency of *Tgfb1* knockout phenotypes

Phenotype	Phenotype penetrance on various strains (%)					
	129 x CF1	129 x C57	129 x C3H	C57	129	C3H 129xC57x NIH/Ola
Preimplantation lethality	50	ND	ND	99	ND	ND 0
Yolk sac lethality*	0	0	ND	ND	ND	ND 50
Autoimmune disease	50	50	50	1	ND	ND 50
Gastric inflammation	90*	20*	ND	ND	ND	ND ND
Intestinal inflammation	0	70*	ND	ND	ND	ND ND
Colon cancer†	ND	ND	ND	ND	100	0 ND

Percentage of knockout animals of a given strain that have the designated phenotype.

*For details, see references 64, 67.

†Approximately 10% of animals with autoimmune disease have no detectable gastrointestinal tract inflammation.

*Unpublished observations.

ND = not determined.

NIH/Ola x C57BL/6 background, the intestines are more severely inflamed than is the stomach (65). Finally, on a predominantly 129 background (129 x CF1; ~97:3), *Tgfb1* knockout mice develop colon cancer if the inflammatory disorder can be eliminated by other genetic manipulations that render the mice immunodeficient (unpublished observations). However, on a predominantly C3H background, immunodeficient *Tgfb1* knockout mice do not develop colon cancer (66). These results suggest that modifier genes exist that can significantly affect the function of TGF β 1 in preimplantation development, yolk sac development, bowel and gastric inflammation, and colon tumor suppression. Progress toward localizing a modifier gene for the yolk sac developmental problem has been made (67).

What is the best genetic background for knockout mice? Because background-dependent phenotypic variability will likely be found for most knockout mice, it will be useful to backcross a targeted allele onto several mouse backgrounds to make congenic strains. In this section, it will be argued that putting a targeted allele on a mixed strain background will also provide useful information. This is not to say that congenic strains are not useful. Rather, the point to be made here is that there also are benefits to looking at mixed strain backgrounds. Again, our experience with *Tgfb* knockout mice will be instructive.

Generating homozygous mutant knockout animals on a mixed genetic background is faster. The ES cells are nearly always from a 129 strain, and the blastocysts into which the targeted ES cells are injected are nearly always C57BL/6. For reasons unknown, this is a good combination for establishing germline transmission of the injected ES cells. The resulting chimeras can then be crossed with any strain desired, but 129, C57BL/6, or Black Swiss mice are most often used, and CF1 mice were used in the case of our *Tgfb1* knockout mice. Heterozygous offspring from this crossing will then be inbred 129 or F1 hybrids of 129 and one of the other strains. Clearly then, the quickest route to having the knockout allele on an inbred strain is through 129. For the other strains several generations of backcrossing is required, which can take well over a year. Unfortunately, strain-129 mice have low fertility and fecundity. Consequently, the number of offspring per litter is usually fewer than six. Although 129 x C57BL/6 hybrids are more robust, upon backcrossing onto C57BL/6, litter size decreases. To the contrary, the Black Swiss and CF1 strains are robust, and litter size often is in excess of 12. The reason for this is probably because they are not truly inbred strains, but

rather are partially outbred through random breeding within their respective strains. Therefore, one of the choices one has is to stay with "pure" genetics at the expense of a lower production rate and considerable delay before generation of experimental animals, or sacrifice some genetic purity to obtain a more efficient production colony. Ideally, one would want to do both, but this often is too expensive.

Mixed genetic background knockout mice often have a wider range of phenotypes. The *Tgfb1* knockout mice backcrossed onto either the 129 or C57BL/6 background (congenics) yield only embryo lethality (63, unpublished observations). On the other hand, when the knockout allele is maintained on mixed genetic backgrounds, embryo and adult phenotypes are maintained.

The *Tgfb2* & *Tgfb3* knockout mice provide further examples. The *Tgfb2* knockout mice have more than two dozen congenital defects and die either immediately preceding or during birth, or within 2 h thereafter (30). Table 2 indicates that most of the phenotypes are only partially penetrant. Though it is not documented, it is likely that the penetrance of some of these phenotypes would increase to nearly 100%, and some of the other phenotypes would disappear were we to put the *Tgfb2* knockout allele on inbred backgrounds. Hence, the mixed strain background probably provided more information than would congenic strains.

The *Tgfb3* knockout mice have a cleft palate (31). One colony of *Tgfb3* knockout mice was left as a mixed background (129 x CF1; 50:50) strain, whereas another colony was backcrossed several generations to the C57BL/6 strain. These two colonies had considerable expressivity differences; the inbred colony had more severe clefting than did the mixed background colony. In the latter, expressivity of clefting varied widely from animal to animal. This variable expressivity within the mixed background colony provided us with the opportunity to obtain far more data on development of the cleft palate and was, therefore, more useful for making assumptions about the cellular and molecular mechanisms by which TGF β 3 supports palate fusion. Hence, using the *Tgfb3* knockout mice, the mixed strain background provided more information than did the congenic strain. Consequently, a wider range of penetrance and expressivity of phenotype is a major advantage of investigating knockout phenotypes in mixed background colonies. Further, variable penetrance of phenotype in a mixed background colony suggests that there are modifier genes for each phenotype that could be obtained by linkage studies.

Conclusions

Questions have been addressed that arose from the last 8 years in which knockout mice have been investigated to analyze gene function at the whole animal level. These questions concern gene redundancy, apparent lack of phenotype in a surprising number of knockout strains, and effects of genetic background on knockout phenotype. Using data obtained principally from *Tgfb* and *Fgf* knockout mice, it is argued that there is probably little redundancy in the genome (i.e., that few genes are dispensable for survival of the species). Apparent lack of phenotype more likely reflects our inability to ask the right questions, or our lack of tools to answer them, than it does a true lack of function. Finally, discussion of genetic background phenotype variability, including variable penetrance and expressivity, was used to present some of the advantages of working with mixed genetic background colonies of knockout mice. For all the examples given here, there are counter examples that must be taken seriously; consequently, these arguments must not be taken as absolutes. For example, if a gene in a particular mouse strain has recently been duplicated, it will most likely be redundant. If one is studying tissue rejection in a knockout mouse, the genetic background obviously must be well defined and preferably inbred. Or, if one wants to use the susceptibility of a particular mouse strain to cancer to investigate the function of the knockout gene in progression of that cancer, the knockout allele must be put on that mouse strain. In general, however, when setting up approaches for investigating a new gene knockout mouse, I believe one would be well advised to assume that: there is little gene redundancy in mammals; there are knockout phenotypes even if none are immediately apparent; and investigating phenotypes in mixed genetic background colonies may not only reveal more phenotypes, but may lead to better understanding of the molecular or cellular mechanism underlying the phenotype, and may lead to modifier gene discovery.

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Front cover Human Genome: Reprinted by permission from *Nature*. International Human Genome Sequencing Consortium. 409:860-921, 2001 © Macmillan Magazines Ltd. Adapted from an image by Francis Collins, NHGRI; Jim Kent, UCSC; Ewan Birney, EBI; and Darryl Leja, NHGRI; showing a portion of Chromosome 1 from the initial sequencing of the human genome.

Back cover In 1967, the British artist Peter Blake created a design classic. Nearly 35 years later Nigel Orme (illustrator), Richard Denyer (photographer), and the authors have together produced an affectionate tribute to Mr Blake's image. With its gallery of icons and influences, its assembly created almost as much complexity, intrigue and mystery as the original. *Drosophila*, *Arabidopsis*, Dolly and the assembled company tempt you to dip inside where, as in the original, "a splendid time is guaranteed for all." (Gunter Blobel, courtesy of The Rockefeller University; Marie Curie, Keystone Press Agency Inc; Darwin bust, by permission of the President and Council of the Royal Society; Rosalind Franklin, courtesy of Cold Spring Harbor Laboratory Archives; Dorothy Hodgkin, © The Nobel Foundation, 1984; James Joyce, etching by Peter Blake; Robert Johnson, photo booth self-portrait early 1930s, © 1986 Delta Haze Corporation all rights reserved, used by permission; Albert L. Lehninger, (unidentified photograph) courtesy of The Alan Mason Cheamey Medical Archives of The Johns Hopkins Medical Institutions; Linus Pauling, from Ava Helen and Linus Pauling Papers, Special Collections, Oregon State University; Nicholas Poush, courtesy of ArtToday.com; Barbara McClintock, © David M. Kios, 1983; Andrei Sakharov, courtesy of Elena Bonner; Frederick Sanger, © The Nobel Foundation, 1958.)

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the target gene in a particular place or at a particular time. The most common of these recombination systems called *Cre/lox*, is widely used to engineer gene replacements in mice and in plants (see Figure 5–82). In this case the target gene in ES cells is replaced by a fully functional version of the gene that is flanked by a pair of the short DNA sequences, called lox sites, that are recognized by the Cre recombinase protein. The transgenic mice that result are phenotypically normal. They are then mated with transgenic mice that express the Cre recombinase gene under the control of an inducible promoter. In the specific cells or tissues in which Cre is switched on, it catalyzes recombination between the lox sequences—excising a target gene and eliminating its activity. Similar recombination systems are used to generate conditional mutants in *Drosophila* (see Figure 21–48).

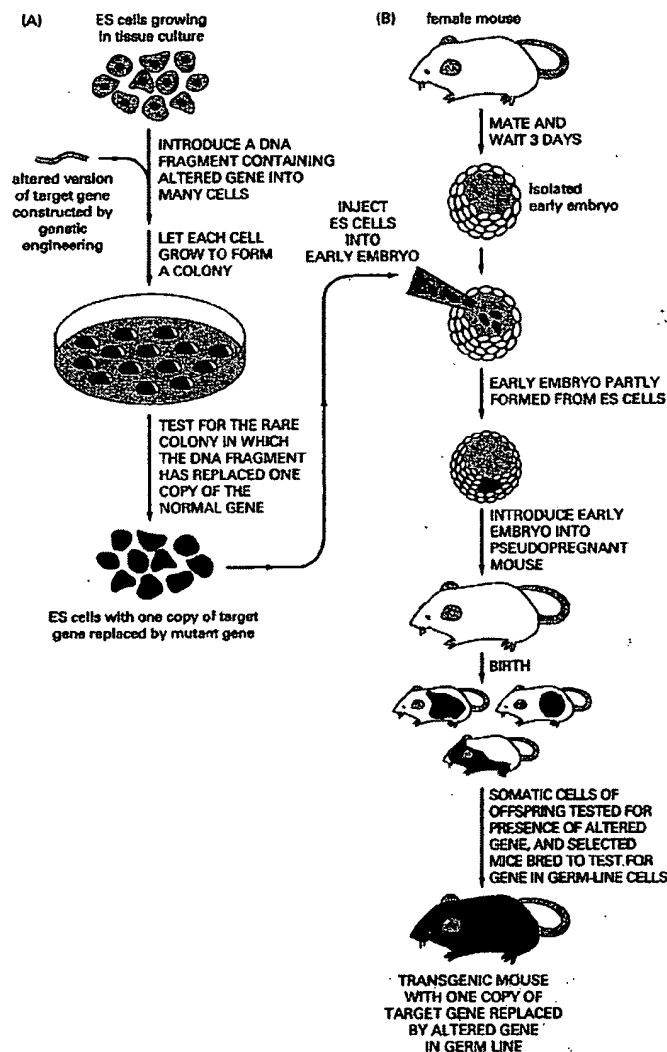


Figure 8–70 Summary of the procedures used for making gene replacements in mice. In the first step (A), an altered version of the gene is introduced into cultured ES (embryonic stem) cells. Only a few rare ES cells will have their corresponding normal genes replaced by the altered gene through a homologous recombination event. Although the procedure is often laborious, these rare cells can be identified and cultured to produce many descendants, each of which carries an altered gene in place of one of its two normal corresponding genes. In the next step of the procedure (B), these altered ES cells are injected into a very early mouse embryo; the cells are incorporated into the growing embryo, and a mouse produced by such an embryo will contain some somatic cells (indicated by orange) that carry the altered gene. Some of these mice will also contain germ-line cells that contain the altered gene. When bred with a normal mouse, some of the progeny of these mice will contain the altered gene in all of their cells. If two such mice are in turn bred (not shown), some of the progeny will contain two altered genes (one on each chromosome) in all of their cells.

If the original gene alteration completely inactivates the function of the gene, these mice are known as knockout mice. When such mice are missing genes that function during development, they often die with specific defects long before they reach adulthood. These defects are carefully analyzed to help decipher the normal function of the missing gene.

Transgenic Plants Are Important for Both Cell Biology and Agriculture

When a plant is damaged, it can often repair itself by a process in which mature differentiated cells "dedifferentiate," proliferate, and then redifferentiate into other cell types. In some circumstances the dedifferentiated cells can even form an apical meristem, which can then give rise to an entire new plant, including gametes. This remarkable plasticity of plant cells can be exploited to generate transgenic plants from cells growing in culture.

When a piece of plant tissue is cultured in a sterile medium containing nutrients and appropriate growth regulators, many of the cells are stimulated to proliferate indefinitely in a disorganized manner, producing a mass of relatively undifferentiated cells called a callus. If the nutrients and growth regulators are carefully manipulated, one can induce the formation of a shoot and then root apical meristems within the callus, and, in many species, a whole new plant can be regenerated.

Callus cultures can also be mechanically dissociated into single cells, which will grow and divide as a suspension culture. In several plants—including tobacco, petunia, carrot, potato, and *Arabidopsis*—a single cell from such a suspension culture can be grown into a small clump (a clone) from which a whole plant can be regenerated. Such a cell, which has the ability to give rise to all parts of the organism, is considered totipotent. Just as mutant mice can be derived by genetic manipulation of embryonic stem cells in culture, so transgenic plants can be created from single totipotent plant cells transfected with DNA in culture (Figure 8-72).

The ability to produce transgenic plants has greatly accelerated progress in many areas of plant cell biology. It has had an important role, for example, in isolating receptors for growth regulators and in analyzing the mechanisms of morphogenesis and of gene expression in plants. It has also opened up many new possibilities in agriculture that could benefit both the farmer and the consumer. It has made it possible, for example, to modify the lipid, starch, and protein storage reserved in seeds, to impart pest and virus resistance to plants, and to create modified plants that tolerate extreme habitats such as salt marshes or water-stressed soil.

Many of the major advances in understanding animal development have come from studies on the fruit fly *Drosophila* and the nematode worm *Caenorhabditis elegans*, which are amenable to extensive genetic analysis as well as to experimental manipulation. Progress in plant developmental biology has, in the past, been relatively slow by comparison. Many of the plants that have proved most amenable to genetic analysis—such as maize and tomato—have long life cycles and very large genomes, making both classical and molecular genetic analysis time-consuming. Increasing attention is consequently being paid to a fast-growing small weed, the common wall cress (*Arabidopsis thaliana*), which has several major advantages as a "model plant" (see Figures 1-46 and 21-107). The relatively small *Arabidopsis* genome was the first plant genome to be completely sequenced.



Figure 8-71 Mouse with an engineered defect in fibroblast growth factor 5 (FGF5). FGF5 is a negative regulator of hair formation. In a mouse lacking FGF5 (right), the hair is long compared with its heterozygous littermate (left). Transgenic mice with phenotypes that mimic aspects of a variety of human disorders, including Alzheimer's disease, atherosclerosis, diabetes, cystic fibrosis, and some type of cancers, have been generated. Their study may lead to the development of more effective treatments. (Courtesy of Gail Martin, from J.M. Hebert et al., *Cell* 78:1017-1025, 1994. © Elsevier.)

Large Collections of Tagged Knockouts Provide a Tool for Examining the Function of Every Gene in an Organism

Extensive collaborative efforts are underway to generate comprehensive libraries of mutations in several model organisms, including *S. cerevisiae*, *C. elegans*, *Drosophila*, *Arabidopsis*, and the mouse. The ultimate aim in each case is to produce a collection of mutant strains in which every gene in the organism has either been systematically deleted, or altered such that it can be conditionally disrupted. Collections of this type will provide an invaluable tool for investigating gene function on a genomic scale. In some cases, each of the individual mutants within the collection will sport a distinct molecular tag—a unique DNA sequence designed to make identification of the altered gene rapid and routine.

In *S. cerevisiae*, the task of generating a set of 6000 mutants, each missing

Genes VII

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which exogenous DNA is introduced from a bacterium into a host cell. The mechanism resembles that of bacterial conjugation. Expression of the bacterial DNA in its new host changes the phenotype of the cell. In the example of the bacterium *Agrobacterium tumefaciens*, the result is to induce tumor formation by an infected plant cell.

Alterations in the relative proportions of components of the genome during somatic development occur to allow insect larvae to increase the number of copies of certain genes. And the occasional amplification of genes in cultured mammalian cells is indicated by our ability to select variant cells with an increased copy number of some genes. Initiated within the genome, the amplification event can create additional copies of a gene that survive in either intrachromosomal or extrachromosomal form.

When extraneous DNA is introduced into eukaryotic cells, it may give rise to extrachromosomal forms or may be integrated into the genome. The relationship between the extrachromosomal and genomic forms is irregular, depending on chance and to some degree unpredictable events, rather than resembling the regular interchange between free and integrated forms of bacterial plasmids.

Yet, however accomplished, the process may lead to stable change in the genome; following its injection into animal eggs, DNA may even be incorporated into the genome and inherited thereafter as a normal component, sometimes continuing to function. Injected DNA may enter the germline as well as the soma, creating a transgenic animal. The ability to introduce specific genes that function in an appropriate manner could become a major medical technique for curing genetic diseases.

The converse of the introduction of new genes is the ability to disrupt specific endogenous genes. Additional DNA can be introduced within a gene to prevent its expression and to generate a null allele. Breeding from an animal with a null allele can generate a homozygous "knockout", which has no active copy of the gene. This is a powerful method to investigate directly the importance and function of a gene.

Considerable manipulation of DNA sequences therefore is achieved both in authentic situations and by experimental fiat. We are only just beginning to work out the mechanisms that permit the cell to respond to selective pressure by changing its bank of sequences or that allow it to accommodate the intrusion of additional sequences.

The mating pathway is triggered by signal transduction

THE yeast *S. cerevisiae* can propagate happily in either the haploid or diploid condition. Conversion between these states takes place by mating (fusion of haploid spores to give a diploid) and by sporulation (meiosis of diploids to give haploid spores). The ability to engage in these activities is determined by the mating type of the strain.

The properties of the two mating types are summarized in Figure 17.1. We may view them as resting on the teleological proposition that there is no point in mating unless the haploids are of different genetic types; and sporulation is productive only when the diploid is heterozygous and thus can generate recombinants.

The mating type of a (haploid) cell is determined by the genetic information present at the *MAT* locus. Cells that carry the *MAT α* allele at this locus are type α ; like-

wise, cells that carry the *MAT α* allele are type α . Cells of opposite type can mate; cells of the same type cannot.

Recognition of cells of opposite mating type is

Figure 17.1 Mating type controls several activities.

	<i>MATα</i>	<i>MATα</i>	<i>MATα/MATα</i>
Cell type	α	α	α/α
Mating	yes	yes	no
Sporulation	no	no	yes
Pheromone	α factor	α factor	none
Receptor	binds α factor	binds α factor	none

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Preface

Over the past ten years it has become possible to make essentially any mutation in the germline of mice by utilizing recombination and embryonic stem (ES) cells. Homologous recombination when applied to altering specific endogenous genes, referred to as gene targeting, provides the highest level of control over producing mutations in cloned genes. When this is combined with site specific recombination, a wide range of mutations can be produced. ES cell lines are remarkable since after being established from a blastocyst, they can be cultured and manipulated relatively easily in vitro and still maintain their ability to step back into a normal developmental program when returned to a pre-implantation embryo. With the exponential increase in the number of genes identified by various genome projects and genetic screens, it has become imperative that efficient methods be developed for determining gene function. Gene targeting in ES cells offers a powerful approach to study gene function in a mammalian organism. Gene trap approaches in ES cells, in particular when they are combined with sophisticated prescreens, offer not only a route to gene discovery, but also to gain information on gene sequence, expression and mutant phenotype.

The basic technology necessary for making designer mutations in mice has become widespread and researchers who have traditionally used cell biology or molecular experiments are adding gene targeting techniques to their repertoire of experimental approaches. A second edition of this book was written for two main reasons. The first was to update previously described techniques and to add new techniques that have greatly expanded the types of mutations that can be made using recombination in ES cells. A chapter in this new edition describes the design and use of site specific recombination for gene targeting approaches and production of conditional mutations. The second reason for the new book was to provide a more in depth discussion of the experimental design considerations that are critical to a successful gene targeting study and to add approaches for analyzing mutant phenotypes, the most interesting part of an experiment. Gene targeting experiments should be designed to go far beyond just making a mutant mouse. The success of a gene targeting experiment no longer lies in the making of the mutation, but depends on the imaginative and insightful analysis of the mutant phenotypes that the mutation provides. A chapter in this edition describes the use of classical genetics in combination with gene targeting to get the most out of a genetic approach to a biological question.

The nature of in vivo gene targeting studies of gene function are such that critical design decisions must be made at every step in the experiment, and each decision can have a major impact on the value of the information obtained. From the start, the type of mutation to be made must be considered

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Preface

carefully. Whereas 10 years ago most mutations were designed to create null mutations and were therefore relatively simple to design, at present, a null mutation is only one of a long list of mutations that can be made, each providing different insight into the function of a gene. Point mutations, large deletions, gene exchanges (knock-ins) and conditional mutations are but a few of the choices one faces at the start of a gene targeting experiment. The next choice is the source of DNA for the targeting experiment and ES cell line to be used for the manipulations. Once the mutant ES cell clone has been obtained, there are then a number of alternative approaches that can be used to make ES cell chimeras that depend on the ES cell line which was used. Finally, and most importantly, is the analysis of any phenotype that arises. This second addition discusses techniques used to analyze mutant mice, ranging from standard descriptive evaluation, to a chimera analysis or complicated breeding experiments that utilize double mutants. If mice are simply considered as a 'bag of cells' or an *in vivo* source of selected cell types, then the tremendous resource which mice offer as a model organism is not being realized. The life of a mouse represents a continuum of dynamic processes, including pattern formation, organ development, learning, homeostasis and disease. By making genetic alterations in mice using gene targeting and ES cells, the effects of a given change can be studied in the context of the whole organism.

My goal in editing this book was to provide a manual that could take a newcomer to the exciting field of gene targeting and mutant analysis in mice from a cloned gene to a basic understanding of the genetic approaches available using ES cells, and how each technique can be used to design a particular *in vivo* test of gene function. The book should also provide a valuable bench side resource for anyone carrying out gene targeting or gene trap experiments, a chimera analysis or classical genetic approaches. I would once again like to extend many thanks and my deepest appreciation to all the authors for their great efforts in including detailed protocols and lucid discussions of the various approaches presented. I would also like to thank my family for their strong support and laboratory members past and present for helping to make gene targeting a reality. Finally, since many of the techniques use mice, the experiments should be carried out in accordance with local regulations.

New York, NY

A.L.J.

Gene targeting, principles, and practice in mammalian cells

PAUL HASTY, ALEJANDRO ABUIN, and ALLAN BRADLEY

1. Introduction

When a fragment of genomic DNA is introduced into a mammalian cell it can locate and recombine with the endogenous homologous sequences. This type of homologous recombination, known as gene targeting, is the subject of this chapter. Gene targeting has been widely used, particularly in mouse embryonic stem (ES) cells, to make a variety of mutations in many different loci so that the phenotypic consequences of specific genetic modifications can be assessed in the organism.

The first experimental evidence for the occurrence of gene targeting in mammalian cells was made using a fibroblast cell line with a selectable artificial locus by Lin *et al.* (1), and was subsequently demonstrated to occur at the endogenous β -globin gene by Smithies *et al.* in erythroleukaemia cells (2). In general, the frequencies of gene targeting in mammalian cells are relatively low compared to yeast cells and this is probably related to, at least in part, a competing pathway: efficient integration of the transfected DNA into a random chromosomal site. The relative ratio of targeted to random integration events will determine the ease with which targeted clones are identified in a gene targeting experiment. This chapter details aspects of vector design which can determine the efficiency of recombination, the type of mutation that may be generated in the target locus, as well as the selection and screening strategies which can be used to identify clones of ES cells with the desired targeted modification. Since the most common experimental strategy is to ablate the function of a target gene (*null allele*) by introducing a selectable marker gene, we initially describe the vectors and the selection schemes which are helpful in the identification of recombinant clones (Sections 2–5). In Section 6, we describe the vectors and additional considerations for generating subtle mutations in a target locus devoid of any exogenous sequences. Finally, Section 7 is dedicated to the use of gene targeting as a method to express exogenous genes from specific endogenous regulatory elements *in vivo*, also known as 'knock-in' strategies.

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1: Gene targeting, principles, and practice in mammalian cells

enrich populations of transfected cells for targeted integration events (Section 4.2.1).

2.1 Design considerations of a replacement vector

The principal consideration in the design of a replacement vector, is the type of mutation generated. Secondary (yet still important) considerations relate to the selection scheme and screening techniques required to isolate the recombinant clones. The recombinant alleles generated by replacement vectors typically have a selection cassette inserted into a coding exon or replacing part of the locus. It is important to consider that, exon interruptions and small deletions will not necessarily ablate the function of the target gene to generate a null allele. Consequently, it is necessary to confirm that the allele which has been generated is null by RNA and/or protein analysis and in many cases transcripts and truncated proteins from such a mutant allele can be detected. Considering that products from the mutated locus may have some function (normal or abnormal) it is important to design a replacement vector so that the targeted allele is null, particularly in the absence of a good assay for the gene product. Disruption or deletion of the coding sequence by the positive selection marker will in most instances ablate a gene's function. However in some situations a truncated protein may be generated which retains some biological activity, thus some knowledge of mutations in a related gene in another organism can be helpful in the determination of the possible function of a targeted allele. Null alleles are more likely to occur by deleting or recombining a selection cassette into more 5' exons rather than exons that encode the C-terminus of the protein, since under these circumstances minimal portions of the wild-type polypeptide would be made.

There are several considerations to take into account when a positive selection marker is to be inserted into an exon. One critical consideration is that since the length of an exon can influence RNA splicing (3), an artificially large exon caused by the insertion of a selectable marker may not be recognized by the splicing machinery and could be skipped. Thus, transcripts initiated from the endogenous promoter may delete the mutated exon from the mRNA species or even additional exons. If a skipped exon is a coding exon whose nucleotide length is not a multiple of three (codon) the net result will be both a deletion and a frame-shift mutation of the gene, which will often generate a null allele. However, if the disrupted coding exon has a nucleotide length which is a multiple of three, if spliced out, this would result in a protein with a small in-frame deletion which may retain partial or complete function. The same concept applies to gene targeting vectors in which exons are being deleted and replaced by the selectable marker. Deletion of an exon or group of exons with a unit number of codons may also result in a functional protein product with an in-frame deletion. For most purposes it is advisable to delete portions or all of the target gene so that the genetic

Production of targeted embryonic stem cell clones

MICHAEL P. MATISE, WOJTEK AUERBACH and ALEXANDRA L. JOYNER

1. Introduction

The discovery that cloned DNA introduced into tissue culture cells can undergo homologous recombination at specific chromosomal loci has revolutionized our ability to study gene function in cell culture and *in vivo*. In theory, this technique, termed gene targeting, allows one to generate any type of mutation in any cloned gene. The kinds of mutations that can be created include null mutations, point mutations, deletions of specific functional domains, exchanges of functional domains from related genes, and gain-of-function mutations in which exogenous cDNA sequences are inserted adjacent to endogenous regulatory sequences. In principle, such specific genetic alterations can be made in any cell line growing in culture. However, not all cell types can be maintained in culture under the conditions necessary for transfection and selection. Over ten years ago, pluripotent embryonic stem (ES) cells derived from the inner cell mass (ICM) of mouse blastocyst stage embryos were isolated and conditions defined for their propagation and maintenance in culture (1, 2). ES cells resemble ICM cells in many respects, including their ability to contribute to all embryonic tissues in chimeric mice. Using stringent culture conditions, the embryonic developmental potential of ES cells can be maintained following genetic manipulations and after many passages *in vitro*. Furthermore, permanent mouse lines carrying genetic alterations introduced into ES cells can be obtained by transmitting the mutation through the germline by generating ES cell chimeras (described in Chapters 4 and 5). Thus, applying gene targeting technology to ES cells in culture affords researchers the opportunity to modify endogenous genes and study their function *in vivo*. In initial studies, one of the main challenges of gene targeting was to distinguish the rare homologous recombination events from more commonly occurring random integrations (discussed in Chapter 1). However, advances in cell culture and in selection schemes, in vector construction using isogenic DNA, and in the application of rapid screening procedures have made it possible to identify homologous recombination events efficiently.

What's Wrong With My Mouse?

*Behavioral Phenotyping of
Transgenic and Knockout Mice*

Jacqueline N. Crawley, Ph.D.

 **WILEY-LISS**

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Preface

Targeted mutation of genes expressed in the nervous system is an exciting new research field that is forging a remarkable amalgam of molecular genetics and behavioral neuroscience. My laboratory in Bethesda has been the fortunate recipient of visits from many molecular geneticists over the past five years, who come to ask, "What's wrong with my mouse? Can you tell us what behaviors are abnormal in our null mutants? And how do you measure behavior, anyway?"

We have had some remarkable opportunities to collaborate with outstanding molecular geneticists in the National Institutes of Health Intramural Research Program and throughout the world on investigations of the behavioral effects of mutations in genes expressed in the mouse brain. Each of these collaborations has been a learning experience, increasing our understanding of the optimal experimental design for analyzing behavioral phenotypes of mutant mice. What are the best tests to address each specific hypothesis? Which methods work best for mice? Which rat tasks can be adapted for mice? What are the correct controls? What are the hidden pitfalls, lurking artifacts, false positives, and false negatives? Which statistical tests are most sensitive for detection of the genotype effect? What is the minimum number of animals necessary for each genotype, gender, and age? Our laboratory and many others are gradually working out the best methods for behavioral phenotyping of transgenic and knockout mice.

In the same conversations, molecular geneticists frequently asked me to recommend a book they could consult to learn more about behavioral tests for mice. Apparently the scientific book publishers are receiving similar queries. Ann Boyle and Robert Harington at John Wiley & Sons, convinced of a real need for such a book, sweet-talked me into filling the void. *What's Wrong With My Mouse?* is written for these pioneering molecular geneticists, and for the talented students who will be the next driving force in moving the field forward.

On a personal level, I would like to express deep appreciation to all of my behavioral neuroscientist colleagues around the world for their outstanding work, past, present, and future. Your contributions to the excellence and abundance of mouse behavioral tests provide

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x PREFACE

the foundation for the rapidly expanding scientific discoveries forthcoming from behavioral phenotyping studies of transgenic and knockout mice. This book is a testament to your accomplishments.

JACQUELINE N. CRAWLEY, Ph.D.
Chesapeake, Maryland

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1

Designer Mice

The disease is inherited. Family pedigrees indicate an autosomal dominant gene. Linkage analyses reveal one strongly associated chromosomal locus. Mapping identifies the gene. The cDNA for the gene is sequenced. The anatomical distribution of the gene is primarily in the brain. The symptoms of the disease are primarily neuropsychiatric. There is no treatment for the disease. The disease is lethal.

Your mission, should you choose to accept it, is to develop a treatment for the disease. Replacement gene therapy is the best hope. But you don't know the gene product, you don't know its function, and you don't know if gene delivery would be therapeutic. Where do you start?

These days, you may choose to start with a targeted gene mutation, to generate a mutant mouse model of the hereditary disease. A DNA construct containing the mutated form of the responsible gene is developed. The construct is inserted into the mouse genome. A line of mice with the mutated gene is generated. Characteristics of the mutant mice are identified in comparison to normal controls. Salient characteristics relevant to the human disease are quantitated. These diseaselike traits are then used as test variables for evaluating the effectiveness of treatments. Putative treatments are administered to the mutant mice. A treatment that prevents or reverses the disease traits in the mutant mice is taken forward for further testing as a potential therapeutic treatment for the human genetic disease. Gene therapy, based on targeted gene replacement of the missing or incorrect gene in the human hereditary disease, is described in Chapter 12. In the future, medicine may shift emphasis from treating the symptoms to administering replacement genes that effectively and permanently cure the disease.

Targeted gene mutation in mice represents a new technology that is revolutionizing biomedical research. Transgenic mice have an *extra gene* added. An additional copy of a normal gene is inserted into the mouse genome to study overexpression of the gene product. Or a new gene is added that is not normally present in the mouse genome. The new gene may be the aberrant form of a human gene linked to a disease. For example, the mutated form of the human *huntingtin* gene is added to the mouse genome to generate a mouse model of Huntington's disease. Knockout mice have a *gene deleted*. The null mutant homozygous

knockout mouse is deficient in both alleles of a gene; the heterozygote is deficient in one of its two alleles for the gene. The genotype is $-/-$ for the null mutant, $+/-$ for the heterozygote, and $+/+$ for the wildtype normal control. The phenotype is the set of observed characteristics resulting from the mutation. Phenotypes include biochemical, anatomical, physiological, and behavioral characteristics.

Targeted mutations of genes expressed in the brain are revealing the mechanisms underlying normal behavior and behavioral abnormalities. Mouse models of human neuropsychiatric diseases, such as Alzheimer's disease, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis, obesity, anorexia, depression, alcoholism, drug addiction, schizophrenia, and anxiety, are likely to be characterized by their behavioral phenotype.

This book is designed to introduce the novice to the rich literature of behavioral tests in mice and to show how to optimize the application of these tests for behavioral phenotyping of mutant mice. Based on our experiences, our laboratory is working toward a unified approach for the optimal conduct of behavioral phenotyping experiments in mutant mice. Recommendations are offered for a three-tiered sequence of behavioral tests, applicable to each behavioral domain relevant to genes expressed in the mammalian brain.

SCOPE

This book is designed as an overview of the mutant mouse technology and an introduction to the field of behavioral neuroscience, as it can be applied to behavioral phenotyping of transgenic and knockout mice. Molecular geneticists may browse through the chapters relevant to their gene, to get ideas for possible tests to try. Behavioral neuroscientists who have no experience with mutant mice may wish to read about the methods for developing a transgenic or knockout, the behavioral tests that have been effectively applied, and some of the successful experiments published in the genetics literature.

Chapters are organized around behavioral domains, including general health, neurological reflexes, developmental milestones, motor functions, sensory abilities, learning and memory, feeding, sexual and parental behaviors, social behaviors, and rodent paradigms relevant to fear, anxiety, depression, schizophrenia, reward, and drug addiction. Each chapter begins with a brief history of the early work in the field and the present hypotheses about mechanisms underlying the expression of the behavior. A list of general review articles and books is offered for each topic, encouraging the interested reader to gain more in-depth knowledge of the relevant literature.

Standard tests are then presented in detail. Highlighted are those tasks that have been extensively validated in mice. Demonstrations of genetic components of task performance are described, including experiments comparing inbred strains of mice (strain distributions), quantitative trait loci approaches (linkage analysis), and naturally occurring mutants (spontaneous mutations). Experimental design and specific behavioral tasks are presented as simply as possible. Extensive references are included for each behavioral test to obtain more complete methods from the primary experimental literature on the topic.

Illustrations are provided for the most frequently used behavioral tasks. Photographs of the equipment or diagrams of the task accompany the text. Samples of data are shown. The data presentation is designed to indicate the qualitative and quantitative results that can be expected when the task is properly conducted.

Each chapter includes the results of several representative experiments in which these tasks are successfully applied to characterize transgenic and knockout mice. Examples are

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WHAT'S WRONG WITH MY MOUSE



Transgenic and knockout mutations provide an important means for understanding gene function, as well as for developing therapies for genetic diseases. This engaging and informative book discusses the many advances in the field of transgenic technology that have enabled researchers to bring about various changes in the mouse genome. Equal emphasis is given to both the principles of transgenic and knockout methods and their applications. A clear and concise format provides researchers with a comprehensive review of the behavioral paradigms appropriate for analyzing mouse phenotypes.

What's Wrong with My Mouse? explains the differences between transgenic knockout mice and their wild-type controls, while providing critical information about gene function and expression. This volume recognizes that newly identified genes can provide useful insights into brain functioning, including brain malfunctioning in disease states. Written by a world-renowned expert in the field, the material also covers:

- How to generate a transgenic or knockout mouse
- Motor functions (open field, holeboard, rotarod, balance, grip, circadian activity, etc.)
- Sensory abilities (olfaction, vision, hearing, taste, touch, nociception)
- Reproductive behavior, social behavior, and emotional behavior

Researchers in neuroscience, pharmacology, genetics, developmental biology, and cell biology will all find this book essential reading.

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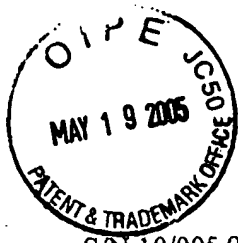
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S/N 10/005,921

PATENT

Confirmation No. 7082

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Keith D. Allen	Examiner:	Shukla, Ram R.
Serial No.:	10/005,921	Group Art Unit:	1632
Filed:	December 4, 2001	Docket No.:	R714/ 75658.022000
Title:	Transgenic Mice Containing CASH Gene Disruptions		

CERTIFICATE UNDER 37 CFR 1.10

'Express Mail' mailing label number: ED888667204US
Date of Deposit: May 19, 2005

I hereby certify that this paper or fee is being deposited with the United States Postal Service 'Express Mail Post Office To Addressee' service under 37 CFR 1.10 on the date indicated above and is addressed to Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

By:

Name: Mary C. Nottel

DECLARATION OF ROBERT DRISCOLL PURSUANT TO 37 C.F.R. § 1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, Robert Driscoll, residing at 23 Chicory Lane, San Carlos, CA 94070, hereby declare:

1. I am presently employed as Vice President of Intellectual Property & Legal Affairs at Assignee, Deltagen, Inc., in San Carlos, CA. I have also previously served as the Company's Senior Director of Intellectual Property, in which position I managed and oversaw the Company's intellectual property portfolio, including the Company's patent filings. I possess a Ph.D in Chemistry, received from the California Institute of Technology. I also possess a J.D., received from Loyola Law School, Los Angeles. I am a registered patent attorney (Reg. No. 47,536).

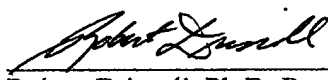
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2. I am familiar with the above-cited application. I am familiar with the Office Action mailed January 11, 2005. I am aware that the Examiner has rejected the claims, in part, for allegedly failing to meet the utility requirement. I am also aware that the Applicant has argued that a commercial sale of a mouse with a disrupted CASH allele within the scope of the claimed subject matter ("CASH gene knockout mouse") should satisfy the utility requirement.

3. In support of the Applicant's aforementioned argument, I hereby state that I have reviewed Deltagen's internal sales records regarding the CASH gene knockout mouse. According to these records, the CASH gene knockout mouse has been delivered to at least one (1) large pharmaceutical company. The contractual terms by which the mice were transferred prohibit Deltagen from identifying the name of this company. However, the company is ranked among the top 10 pharmaceutical companies worldwide (based on sales).

4. It is my understanding, based on communications with our pharmaceutical company customers, that transgenic knockout mice obtained from Deltagen are used for studying gene function and for human therapeutic drug development.

5. I further declare that all statements made herein of my own knowledge are true; and further that these statements were made with knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the above-referenced application or any patent issuing thereon.


Robert Driscoll, Ph.D, Reg. No. 47,536

2 May 2005
Date

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S/N 09/903,393

PATENT

Confirmation No. 9468

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Keith D. Allen	Examiner:	Shukla, Ram R.
Serial No.:	10/005,921	Group Art Unit:	1632
Filed:	December 4, 2001	Docket No.:	R714/75658.022000
Title:	Transgenic Mice Containing CASH Gene Disruptions		

DECLARATION OF JOHN BURKE PURSUANT TO 37 C.F.R. § 1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, John E. Burke, residing at 16357 E. Berry Avenue, Centennial CO 80015, hereby declare:

1. I am currently, and have been since 1998, the Attorney of Record for the Applicant and Assignee, Deltagen, Inc. I am listed on the originally filed Power of Attorney for the present application. From December 1996 to December 1999, I was Of Counsel with the law firm of Pillsbury Madison & Sutro (currently Pillsbury Winthrop), where I represented Deltagen with respect to intellectual property matters, including patent matters relating to their transgenic mouse program. From December 1999 until December 2001, I served as Deltagen's Vice President of Intellectual Property, where I supervised Deltagen's internal patent department. All of the applications, including the present application, covering the 750 lines of mice in DeltaBase were drafted by Deltagen's patent department. From December 2001 until April 2003, I served as Deltagen's Senior Vice President and General Counsel. From April 2003 through April 2005, I was a partner with the Denver office of Merchant & Gould, where I continued to represent Deltagen with regard to intellectual property matters, including patent matters. I am presently employed as a Shareholder with the Denver office of the law firm of Greenberg Traurig, where I

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am responsible for prosecution of Deltagen's patent portfolio relating to their transgenic Mouse program, including the present application.

2. I am familiar with the present application, as well as the Office Actions mailed January 11, 2005 and July 28, 2004. I am aware that the Examiner has rejected the claims, in part, for allegedly failing to meet the utility and enablement requirements. I am aware that the Examiner argues on page 4 of the January 11, 2005 Office Action that the reported phenotypes "cannot reliably contribute to disruption in CASH gene." The Examiner further argues on page 5 that the Applicant "has not provided any substantial evidence to obviate the grounds of rejection set forth in the previous office actions or this office action and that CASH gene is associated with any disease or condition or that seizure or pain or other phenotype as claims or disclosed i[s] specific to CASH gene disruption." I am aware that the Examiner argues on page 3 of the July 28, 2004 "that the phenotype of pain varies even among mice of different genetic backgrounds. Applicants' arguments that Crabbe reference fails to establish that phenotypic differences between a knock out transgenic mouse and wild-type control mouse are not real are besides the point because applicants have to provide evidence that the phenotype of the claimed transgenic mouse is due to disruption of CASH gene."

3. I hereby declare that, as evidenced by the attached Exhibit, the subject matter of the present application, CASH gene knockout mice, were compared with control mice of identical background. The data show that there is a statistically significant difference between the results of the Hot plate test, used to measure pain sensitivity, and the Metrazol test, used to measure susceptibility to seizures. The results show that the observed phenotypes are in fact a result of the gene disruption.

4. I hereby declare that the claimed CASH gene knockout mouse has been extensively analyzed using the tests set forth in the Examples. This data has been incorporated into Deltagen's commercial database product, DeltaBase. This database has been subscribed to by at least three of the world's largest pharmaceutical companies, Merck, Pfizer and GSK.

5. I hereby declare that I have accessed Deltagen's internal web-based DeltaBase database to review the data derived from analyses of the claimed mice. I hereby declare that the attached Exhibit contains four (4) pages, each representing a screen printout from DeltaBase.

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The first page is the Behavior Summary page summarizing changes relating to genotype associated with Gene 714, as prepared by Deltagen's pathology group. As noted at the top of the page, Gene 714 corresponds to the CASH gene knockout mouse. As noted, the heterozygous mice showed a decrease in their response to the Hot plate test, relative to wild-type controls. The heterozygous mice also displayed a decreased response threshold on the Metrazol test. The page further notes that for the behavioral tests, 12 heterozygous mutant males were compared with 12 wild-type controls males. The page further describes how 129/OlaHsd x C57BL/6 F2N1 mice were produced. The table on the right side of the page provides the background of each of the heterozygous (-/-) and wild-type control mice (+/+) used in the comparative tests. As shown in the table, the transgenic mice (-/+) and control mice (+/+) are of identical F2N1 background (129/OlaHsd x C57BL/6).

6. The second page shows the raw data derived from the comparative Hot plate tests for Gene 714. The gene number, 714, is indicated in column 1. The ES cell line is indicated in column 2. As can be seen, each mouse was derived from the same ES cell line, 1078. Columns 3 and 4 indicate the generation and background. As can be seen, each mouse is of identical background and generation, F2N1. As can also be seen, each mouse tested is of the same age and gender. Column 13 shows the date on which the data was recorded. The majority of the data corresponding to the F2N1 mice was entered in November and December of 2000.

7. The third page shows the raw data derived from the comparative Metrazol tests for Gene 714. The gene number, 714, is indicated in column 1. The ES cell line is indicated in column 2. As can be seen, each mouse was derived from the same ES cell line, 1078. Columns 3 and 4 indicate the generation and background. As can be seen, each mouse is of identical background and generation, F2N1. As can also be seen, each mouse tested is of the same age and gender. Column 13 shows the date on which the data was recorded. The majority of the data corresponding to the F2N1 mice was entered in November and December of 2000.

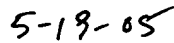
8. The fourth page of the Exhibit shows the average and standard deviation of the Hot plate test and Metrazol test for Gene 714 (rows 17-19). As shown, the statistical analyses for the Hot plate test and Metrazol test were based on eleven (11) controls and eleven (11) transgenic mice. As indicated in column 11, the results were statistically significant.

8. In summary, the attached Exhibits show that the transgenic mice were compared with control mice of identical background. The phenotypes were based on a comparison with age, gender and strain matched control mice.

9. I further declare that all statements made herein of my own knowledge are true; and further that these statements were made with knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the above-referenced application or any patent issuing thereon.



John E. Burke, Reg. No. 35,836



Date

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Gene: 714 Name: Cflar Family: Protease Subfamily: Caspase Alternative Names

Nucleotide Sequence Accession: Y14041 GI: 2653419 External Links: Select External Database

Last Modified By: Kash, Shera
Last Modified On: 9/15/2001 1:20:26 PM

Gene 714
Behavior

Changes related to genotype:

- Heterozygous mutants displayed a decrease in response time on the Hot Plate test.
- Heterozygous mutant mice displayed a decreased response threshold on the Metrazol test.

Heterozygous mutant and wild-type control mice were evaluated for phenotypic changes by testing on six behavioral tasks: Open field test, Tail suspension test, Rotarod test, Hot plate test, Startle/PPI, and Metrazol test.

Mouse ID numbers are as follows:

12 heterozygous mutant males (112686, 119888, 98943, 98933, 98942, 112698, 118189, 98937, 112695, 98930, 127141, 103817)

12 wild-type control males (98944, 98940, 112667, 98939, 103815, 98935, 112672, 112693, 118188, 119889, 127139, 98936)

ES cells derived from the 129/OlaHsd mouse substrain were used to generate chimeric mice. F1 mice were generated by breeding with C57BL/6 females. The resultant F1N0 heterozygotes were backcrossed to C57BL/6 mice to generate F1N1 heterozygotes. F2N1 heterozygous mutant mice were produced by intercrossing F1N1 heterozygous males and females.

Behavior Findings:

When compared to age- and gender-matched wild-type control mice, heterozygous mutant mice responded significantly faster on the Hot Plate test, indicating that they may have increased pain sensitivity.

Mice

#	Sex	Genotype	F Gen.	N Gen.	Age	Validity	Release
98930	Male	-/+	2	1	68	V	T
98930	Male	-/+	2	1	81	V	T
98933	Male	-/+	2	1	68	V	T
98933	Male	-/+	2	1	74	V	T
98937	Male	-/+	2	1	68	V	T
98937	Male	-/+	2	1	75	V	T
98942	Male	-/+	2	1	70	V	T
98943	Male	-/+	2	1	70	V	T
98943	Male	-/+	2	1	76	V	T
103817	Male	-/+	2	1	72	V	T
103817	Male	-/+	2	1	82	V	T
112686	Male	-/+	2	1	70	V	T
112686	Male	-/+	2	1	80	V	T
112695	Male	-/+	2	1	71	V	T
112695	Male	-/+	2	1	81	V	T
112698	Male	-/+	2	1	71	V	T
112698	Male	-/+	2	1	81	V	T
118189	Male	-/+	2	1	70	V	T
118189	Male	-/+	2	1	79	V	T
119888	Male	-/+	2	1	73	V	T
119888	Male	-/+	2	1	83	V	T
127141	Male	-/+	2	1	72	V	T
127141	Male	-/+	2	1	81	V	T
98935	Male	+/+	2	1	68	V	T
98935	Male	+/+	2	1	81	V	T
98936	Male	+/+	2	1	68	V	T
98936	Male	+/+	2	1	75	V	T
98939	Male	+/+	2	1	70	V	T
98939	Male	+/+	2	1	76	V	T
98940	Male	+/+	2	1	70	V	T
98944	Male	+/+	2	1	70	V	T
103815	Male	+/+	2	1	72	V	T
103815	Male	+/+	2	1	89	V	T
112667	Male	+/+	2	1	70	V	T
112667	Male	+/+	2	1	80	V	T
112672	Male	+/+	2	1	70	V	T
112672	Male	+/+	2	1	80	V	T
112693	Male	+/+	2	1	71	V	T
112693	Male	+/+	2	1	81	V	T
118188	Male	+/+	2	1	70	V	T
118188	Male	+/+	2	1	79	V	T
119889	Male	+/+	2	1	73	V	T
119889	Male	+/+	2	1	83	V	T

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ES Cell										Time at		Test		Working		ES Cell	
Gene	Line	F #	N #	Genotype	Gender	Age at Test (days)	Mouse temperature (C)	licking (s)	Validity	Release Flag	Test Date	Test Id	Scientist	Set	Line Order		
714	1078	1	0	+/+	Male	81	82723	55.5	V	F	9/20/2000 1:41:36 PM	3947949	Phu Tran	DB2	1		
714	1078	1	0	+/+	Male	81	82727	55.5	V	F	9/20/2000 1:42:37 PM	3947970	Phu Tran	DB2	1		
714	1078	1	0	+/+	Male	81	82728	55.5	V	F	9/20/2000 1:43:33 PM	3947990	Phu Tran	DB2	1		
714	1078	1	0	+/+	Male	81	82730	55.5	V	F	9/20/2000 1:44:35 PM	3947999	Phu Tran	DB2	1		
714	1078	2	1	+/+	Male	73	98935	55.5	V	T	11/1/2000 5:17:08 PM	5394438	Phu Tran	DB2	1		
714	1078	2	1	+/+	Male	73	98936	55.5	V	T	11/1/2000 5:18:00 PM	5394443	Phu Tran	DB2	1		
714	1078	2	1	+/+	Male	75	98939	55.5	V	T	11/1/2000 5:12:14 PM	5394418	Phu Tran	DB2	1		
714	1078	2	1	+/+	Male	82	103815	55.5	V	T	11/30/2000 2:11:13 PM	7226974	Todd Westergard	DB2	1		
714	1078	2	1	+/+	Male	79	112667	55.5	V	T	12/27/2000 4:07:56 PM	8113403	Phu Tran	DB2	1		
714	1078	2	1	+/+	Male	79	112672	55.5	V	T	12/27/2000 4:12:56 PM	8113532	Phu Tran	DB2	1		
714	1078	2	1	+/+	Male	80	112693	55.5	V	T	12/27/2000 3:46:24 PM	8113196	Phu Tran	DB2	1		
714	1078	2	1	+/+	Male	79	118188	55.5	V	T	1/17/2001 3:27:37 PM	8469367	Sara Siebel	DB2	1		
714	1078	2	1	+/+	Male	82	119889	55.5	V	T	1/31/2001 4:57:57 PM	8824381	Phu Tran	DB2	1		
714	1078	2	1	+/+	Male	81	127139	55.5	V	T	3/1/2001 4:10:53 PM	9988783	Todd Westergard	DB2	1		
714	1078	2	1	-/-	Male	73	98930	55.5	V	T	11/1/2000 5:15:36 PM	5394428	Phu Tran	DB2	1		
714	1078	2	1	-/-	Male	73	98933	55.5	V	T	11/1/2000 5:16:25 PM	5394433	Phu Tran	DB2	1		
714	1078	2	1	-/-	Male	73	98937	55.5	V	T	11/1/2000 5:18:45 PM	5394448	Phu Tran	DB2	1		
714	1078	2	1	-/-	Male	75	98943	55.5	V	T	11/1/2000 5:13:06 PM	5394423	Phu Tran	DB2	1		
714	1078	2	1	-/-	Male	82	103817	55.5	V	T	11/30/2000 2:11:23 PM	7226977	Todd Westergard	DB2	1		
714	1078	2	1	-/-	Male	79	112686	55.5	V	T	12/27/2000 3:38:41 PM	8113047	Phu Tran	DB2	1		
714	1078	2	1	-/-	Male	80	112695	55.5	V	T	12/27/2000 3:47:35 PM	8113222	Phu Tran	DB2	1		
714	1078	2	1	-/-	Male	80	112698	55.5	V	T	12/27/2000 3:48:27 PM	8113246	Phu Tran	DB2	1		
714	1078	2	1	-/-	Male	79	118189	55.5	V	T	11/7/2001 3:29:26 PM	8469372	Sara Siebel	DB2	1		
714	1078	2	1	-/-	Male	82	119888	55.5	V	T	1/31/2001 4:57:12 PM	8824376	Phu Tran	DB2	1		
714	1078	2	1	-/-	Male	81	127141	55.5	V	T	3/1/2001 4:11:14 PM	9988786	Todd Westergard	DB2	1		

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ES Cell		Dose at						Test		Working		ES C						
Gene	Line	F #	N #	Genotype	Gender	Age at Test	Mouse	first twitch	clonic seizure	tetanic extension	death	Validity	Release Flag	Test Date	Test Id	Scientist	Set	Line Or
								(mg/kg)										
714	1078	1	0	+/-	Male	81	82723	26.502	33.127	119.258	143.551	V	F	9/20/2000 1:52:06 PM	3948074	Phu Tran	DB2	1
714	1078	1	0	+/-	Male	81	82727	25.183	32.051	113.324	151.099	V	F	9/20/2000 1:53:53 PM	3948108	Phu Tran	DB2	1
714	1078	1	0	+/-	Male	81	82730	25.091	50.182	124.316	157.391	V	F	9/20/2000 1:55:58 PM	3948160	Phu Tran	DB2	1
714	1078	2	1	+/-	Male	81	98935	27.083	32.292	111.458	131.250	V	T	11/9/2000 3:25:43 PM	6383552	Sara Siebel	DB2	1
714	1078	2	1	+/-	Male	75	98936	14.254	25.219	127.193	154.605	V	T	11/3/2000 3:37:24 PM	6262260	Phu Tran	DB2	1
714	1078	2	1	+/-	Male	76	98939	26.141	40.399	120.010	139.021	V	T	11/2/2000 3:30:25 PM	6262206	Phu Tran	DB2	1
714	1078	2	1	+/-	Male	89	103815	22.096	32.618	124.158	143.098	V	T	12/7/2000 3:36:21 PM	7699422	Todd Westergard	DB2	1
714	1078	2	1	+/-	Male	80	112667	21.895	27.866	92.556	120.422	V	T	12/28/2000 5:51:24 PM	8114212	Phu Tran	DB2	1
714	1078	2	1	+/-	Male	80	112672	29.183	41.342	119.163	148.346	V	T	12/28/2000 5:52:27 PM	8114227	Phu Tran	DB2	1
714	1078	2	1	+/-	Male	81	112693	23.408	49.157	136.938	179.073	V	T	12/28/2000 5:47:22 PM	8114167	Phu Tran	DB2	1
714	1078	2	1	+/-	Male	79	118188	19.330	28.995	114.905	147.122	V	T	1/17/2001 3:35:18 PM	8469427	Sara Siebel	DB2	1
714	1078	2	1	+/-	Male	83	119889	28.045	57.425	140.224	168.269	V	T	2/1/2001 4:05:29 PM	8876813	Phu Tran	DB2	1
714	1078	2	1	+/-	Male	81	127139	26.961	57.598	155.637	175.245	V	T	3/1/2001 4:15:14 PM	9989873	Todd Westergard	DB2	1
714	1078	2	1	-/+	Male	81	98930	22.410	43.576	117.032	151.892	V	T	11/9/2000 3:25:07 PM	6383537	Sara Siebel	DB2	1
714	1078	2	1	-/+	Male	74	98933	26.502	33.127	109.320	142.447	V	T	11/2/2000 3:33:26 PM	6262236	Phu Tran	DB2	1
714	1078	2	1	-/+	Male	75	98937	35.714	40.816	125.000	147.959	V	T	11/3/2000 3:38:19 PM	6262275	Phu Tran	DB2	1
714	1078	2	1	-/+	Male	76	98943	39.370	40.600	52.904	71.358	V	T	11/2/2000 3:31:31 PM	6262221	Phu Tran	DB2	1
714	1078	2	1	-/+	Male	82	103817	26.042	43.025	61.141	89.447	V	T	11/30/2000 2:25:13 PM	7227124	Todd Westergard	DB2	1
714	1078	2	1	-/+	Male	80	112686	29.819	39.361	58.445	96.613	V	T	12/28/2000 5:41:25 PM	8114152	Phu Tran	DB2	1
714	1078	2	1	-/+	Male	81	112695	31.888	36.139	107.355	127.551	V	T	12/28/2000 5:49:03 PM	8114182	Phu Tran	DB2	1
714	1078	2	1	-/+	Male	81	112698	29.332	39.486	100.406	127.482	V	T	12/28/2000 5:49:58 PM	8114197	Phu Tran	DB2	1
714	1078	2	1	-/+	Male	79	118189	18.008	23.305	88.983	116.525	V	T	1/17/2001 3:36:03 PM	8469442	Sara Siebel	DB2	1
714	1078	2	1	-/+	Male	83	119888	24.671	81.209	84.293	104.852	V	T	2/1/2001 4:03:50 PM	8876790	Phu Tran	DB2	1
714	1078	2	1	-/+	Male	81	127141	26.355	38.906	133.032	161.898	V	T	3/1/2001 4:16:14 PM	9989891	Todd Westergard	DB2	1

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Gene	Line	ES Cell	F #		Age	Bin	Genotype	Gender	Study		Statistic	Statistic Value	Mouse Count (n)
									Procedure	Name			
714	1078	2	1	49	-/+			Female	serum chemistry		1-p value vs. WT controls	1	3
714	1078	2	1	49	-/+			Female	serum chemistry	chloride	1-p value vs. WT controls	0.98	3
714	1078	2	1	90	-/+			Female	hematology	aspartate transferase	1-p value vs. WT controls	0.96	4
714	1078	2	1	90	-/+			Female	hematology	white blood cells	1-p value vs. WT controls	0.97	4
714	1078	2	1	90	-/+			Female	hematology	absolute lymphocytes	1-p value vs. WT controls	0.96	4
714	1078	2	1	90	-/+			Female	hematology	absolute monocytes	1-p value vs. WT controls	0.97	4
714	1078	2	1	90	-/+			Female	hematology	absolute eosinophils	1-p value vs. WT controls	0.96	4
714	1078	2	1	90	-/+			Female	hematology	segmented neutrophils	1-p value vs. WT controls	0.98	4
714	1078	2	1	90	-/+			Female	serum chemistry	albumin	1-p value vs. WT controls	0.97	4
714	1078	2	1	180	-/+			Female	serum chemistry	potassium	1-p value vs. WT controls	0.99	3
714	1078	2	1	49	-/+			Male	hematology	platelets	1-p value vs. WT controls	0.99	3
714	1078	2	1	49	-/+			Male	serum chemistry	blood urea nitrogen	1-p value vs. WT controls	0.96	3
714	1078	2	1	49	-/+			Male	serum chemistry	creatinine kinase - total	1-p value vs. WT controls	0.95	3
714	1078	2	1	49	-/+			Male	serum chemistry	osmolality	1-p value vs. WT controls	0.99	11
714	1078	2	1	49	-/+			Male	necropsy weights	liver / body weight	1-p value vs. WT controls	0.99	11
714	1078	2	1	49	-/+			Male	startle	P110, standard deviation	1-p value vs. WT controls	0.99	11
714	1078	2	1	49	-/+			Male	startle	PPI80, standard deviation	1-p value vs. WT controls	0.98	11
714	1078	2	1	49	-/+			Male	hot plate	latency to hindpaw licking	1-p value vs. WT controls	0.99	11
714	1078	2	1	49	-/+			Male	metrazol	latency to tonic extension	1-p value vs. WT controls	0.99	11
714	1078	2	1	49	-/+			Male	metrazol	latency to death	1-p value vs. WT controls	0.99	11

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